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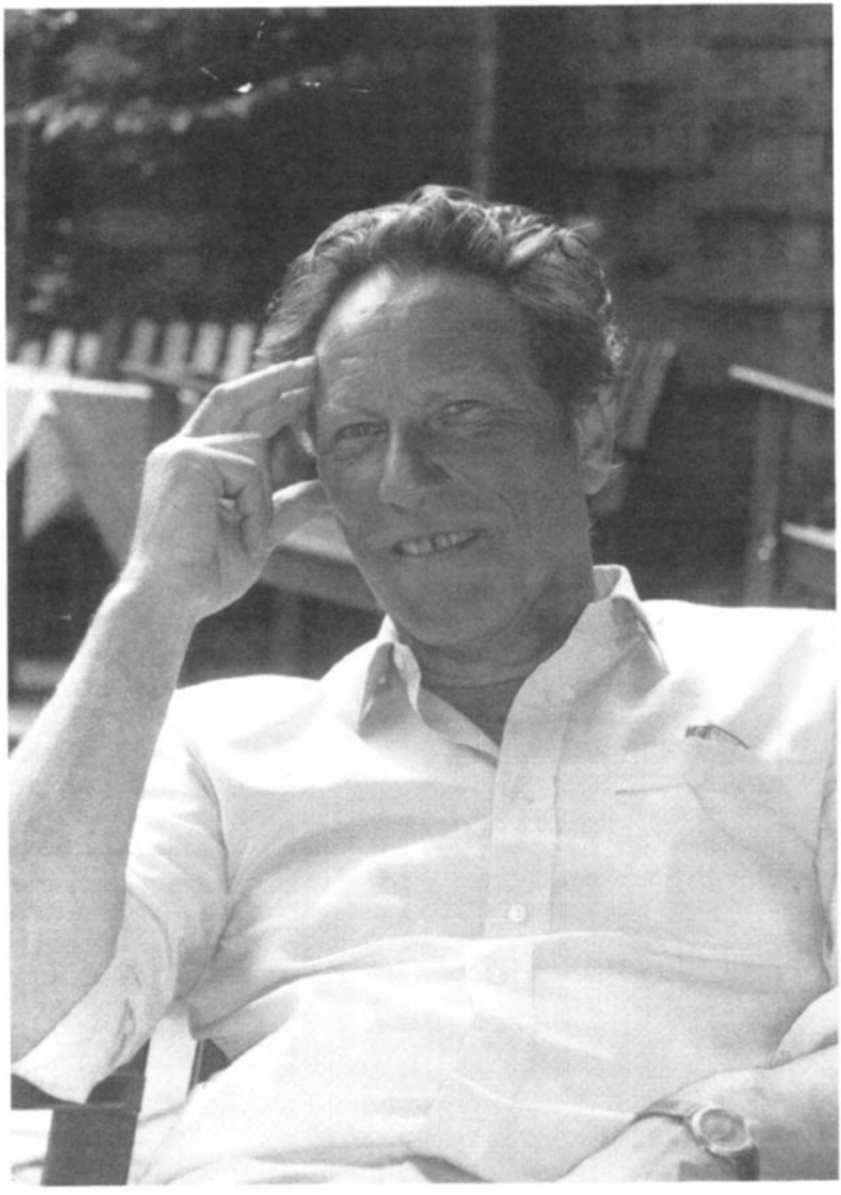

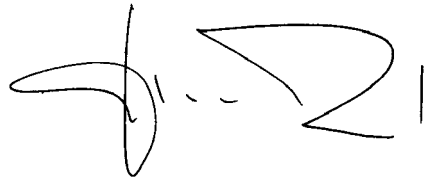

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HLA AND I

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Introduction

The title of this prefatory chapter is borrowed from *The Egg and I*, a story describing life on a chicken farm (1). This resemblance is not accidental. In my life I have found myself often in comparable situations: that is, trying to keep afloat in the midst of many activities, over the majority of which I did not have much control. Looking back I realize how little of my life was planned, and how much was due to chance or luck, good or bad.

How Did I Get There in the First Place?

One of the many events outside my control that had a major influence on my life was an "otitis media" for which I was hospitalized at the tender age of 18 months. By the time I was cured I had been operated on four times, and the hospital had become a very familiar place; that certainly played a role in my choice of a medical career.

The beginning of my medical studies in 1944 was frustrated by the German occupation. The universities were closed, and after having been imprisoned twice (once released and once escaped), I was in hiding. The winter of 1944–1945 was bitterly cold. There was no heating, no light, and very little food. I sat long days with two overcoats, cap, gloves, and my feet in a foot-muff studying basic physics, chemistry, zoology, and a little bit of anatomy. I managed to pass my exam in chemistry: the examiner was the father of a schoolmate of mine. Professor van Arkel, disguised by a mustache, long hair, and another name, was in hiding too.

That winter might have been the end of a promising career as well, if my girlfriend of the time had not had her birthday party. In hiding, of

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course, I could not attend her party and instead was trying to study while feeling sorry for myself, sitting before a south-facing window to catch whatever sunshine there was. After an hour or so, I decided: "To hell with hiding"; I walked through gardens of our neighbors, avoiding streets as much as possible, to join the fun. I was enjoying a "real" cup of tea and even a cookie when an enormous explosion occurred nearby: one of the V2 missiles aimed at London had backfired and hit the ground half a mile away. When I returned to my room I found the window smashed to pieces. One swordlike part penetrated the back of my chair. This shows how important the birthday party of a girlfriend can be.

Discovering Science

I had the privilege to be brought up in both academic (my father was an engineer) and artistic surroundings (my mother was a musician, my stepfather a portrait painter). One of the good things during the war was that people organized lectures, recitals, and chamber music in their homes. I loved these and learned probably as much that way as at school although we had some outstanding teachers there as well (Schulte Nordholt, Staverman, Huygens, Ousoren).

Directly after the war when I had turned 19 years old, I became a student in Leiden, where I spent almost my entire life. During my studies I had ample opportunity to get acquainted not only with the medical and biological sciences, but also with a bit of philosophy.

After the war the facilities of the University left much to be desired, although they must have been splendid in comparison to those found by George Klein in Budapest in 1945 (2). We had our lectures in cinema theatres; the backlog of students was so great that the lecture halls could not contain them. Student social life was thriving and I made some friends for a lifetime there.

To compensate for the massive ex cathedra lectures, numerous debating groups were started under the guidance of senior faculty members. I joined the discussion group "Morula" with Professor Piet Gaillard as mentor. Gaillard, a histologist, was one of the most brilliant scientists of the Leiden Medical Faculty of that time. Although the emphasis was on preclinical studies, my first encounter with transplantation occurred when Gaillard told us of a patient who had had a successful parathyroid allotransplant. Gaillard's main scientific interest was bone formation, and he had developed sophisticated bone and parathyroid culture facilities and techniques. When, during the war, a girl who had undergone a partial thyroidectomy became hypoparathyroidal, it was realized that her parathyroid glands had been removed as well. What to do? There was no parathyroid hormone available. The girl had life threatening seizures

caused by hypocalcinemia. Gaillard and Kooreman, the surgeon, decided to transplant several of the parathyroid clusters Gaillard had had in culture for several weeks. An allogenic transplantation avant la lettre! The tissue cultures were placed in the perivascular lymph space of one of the axillary veins. The girl recovered in a few days and went home. A year later she was run over by a tram in front of the hospital and brought to the emergency room, where she died. At autopsy normal parathyroid tissue was found (3)!

This was thirty years before Lafferty demonstrated the same for islet transplantation, and others made similar, less reproducible, claims. During the discussion I asked why transplants normally were rejected. Gaillard said he did not know and that it had probably something to do with the nucleus [not bad!], but mainly with his excellent tissue culture technique [even better!]. This was in 1946, the year in which, unknown to me at the time, Medawar published his classic paper in the *British Journal of Experimental Pathology* on skin transplant rejection and the ability of leukocytes to induce a second set rejection. This publication to a large extent formed the basis of my scientific life (4).

A Beginners Course in Management

The organization of student life in the Netherlands was and still is rather diverse. It varies from no organization at all: you simply stay home with your parents (not to be preferred but very economical) to becoming a member of one of the fraternities. For the one I joined the newcomers customarily organized themselves in clubs of about 25 people. I was lucky in that the club I joined has remained over four decades a close knit group. We still meet regularly. Several of our members were socially quite active and through them I got involved in management. Without doubt the culmination of this was when I was put in charge of a small refugee camp housed in one of the mansions on the grounds of Zuylen, a castle near Utrecht. When I married Sacha van Tuyll ten years later, I found out that her family were the original owners of the estate.

Discovering Medicine

In the 1940s the study of medicine in Holland was strictly separated into preclinical (first three years) and clinical (four years) phases. The introduction to clinical medicine took place not on the wards, but in the lecture halls. A patient was rolled in and discussed often not once but on two or even three different days. By far the best teacher of clinical medicine was Jaap Mulder, professor in internal medicine. Not only did he introduce us in an elegant manner to the intricacies of history taking and physical examination, he also taught us how to approach a patient. He was medium

sized with a pronounced chin and equally pronounced opinions. The many anecdotes about him all told of a strong, independent man, who accepted no restrictions on his activities if he thought they were necessary for the welfare of his patients. He was often in conflict with the hospital and the health insurance authorities. He always won and he was our hero. Mulder had been a general practitioner in Indonesia (then still the Netherlands East-Indies) and taught us how to become good general practitioners: "no fuss, no high tech, use your brain and your hands."

I might well have become a general practitioner if my girl friend had stayed in Holland. She went to New York, however, got a job, was lonely, and asked me to come over. That was easier said than done. To get into the United States in the 1950s you needed not only money, which I didn't have, but also a visa, which you couldn't get if you had no sponsor who could take care of you financially. My parents had just been to the States for a short visit and on the boat to New York had met Professor Bloch, then working at the Rockefeller Institute. When told that I studied medicine, he made the "mistake" of offering help if I ever wanted to come to the United States. He forgot about it, but my mother didn't. On her suggestion I wrote Bloch, explaining my low status as a medical student who had just finished (with some difficulty, but I don't think I mentioned that) the theoretical part of my medical education and asking his help. To my surprise he answered me by return mail: "Dear Dr. van Rood, we are so pleased that you are willing to come to New York. Please let me know whether you want to work at Bellevue Hospital or Presbyterian Hospital." Clearly there had been a misunderstanding! I wrote back, explaining that I had had no clinical experience or responsibility. When Bloch in a second letter reiterated his invitation (and my girlfriend became insistent) I thought, "Why not give it a try?" So I booked on the Groote Beer and sailed to Quebec. Once arrived I took a Greyhound bus and presented myself to Professor Bloch. When it finally dawned on him that I was not a physician, he looked somewhat taken aback, but he sent me with an introductory letter to Robert Loeb, professor of internal medicine at the Presbyterian Hospital.

Hearing the reason of my visit Miss Palmer, secretary to Professor Loeb, exclaimed: "What a coincidence! Only two days ago we had a visit from such a nice young doctor from Holland—Dr. Querido." He was the second professor in medicine in Leiden. Life was confusing! Loeb was even more distinguished and more taken aback than Bloch when he realized that I was not a physician. After some hesitation he accepted me as a clinical clerk; which sounded wonderful but was in fact quite tough. We started at 7 AM, had teaching rounds each morning, classes in the afternoon, and the library in the evening. Loeb who had written the classic textbook on

Medicine together with Cecil was a superb teacher and a true clinical scientist.

He had collected around him physicians of equal status such as Hanger and others. I was awestruck and delighted. In all probability this period programmed me to become an (academic) internist. It was here that for the first time I had the (primary) responsibility for my own patients, such as Mary, a young girl with IDDM, J. R. with a hepatoma, G. L. with heart failure, and also Billy, a small time gangster who, if he got in a squeeze, put red ink in his urine so that he would be admitted to the safe haven of the hospital. Another "patient" was John an alcoholic who donated blood (for which he was paid) to obtain the cash to buy whisky. When he became so anemic that he collapsed on the street, he was brought into the hospital where he was given blood transfusions. My first encounter with blood transfusion medicine! Listening to Loeb's recollections of the first patient with pernicious anaemia who was saved by a diet of raw liver made one realize the challenge and the adventure that clinical science is.

After my return to Holland I started my internships. During my attending on the obstetric wards for the second time and in a dramatic way I confronted blood transfusion medicine. One of my patients received 24 units of blood for post partum bleeding. In those days this was a heroic effort of which no one had much experience. We tried to stop the bleeding and control the electrolyte balance, without avail however and the patient died. I had hoped to get a consultation from the department of internal medicine, but because of my lowly position as an intern and probably also because the professor of obstetrics was not on the best terms with his colleague in the department of internal medicine, this did not happen. At the autopsy I raised the point of the missed consultation again. The next morning I was called by the Professor of Obstetrics, Holmer, a rather authoritarian man, and was told that because I criticized the decisions made in his department, I obviously had nothing to learn and could go home. It would have meant the end of my medical career. I did not like the idea, but neither did I like backing out of what was my sincere medical opinion. We had what is called a frank discussion, and I was allowed to continue my studies. The sequelae of pregnancy and the role of blood transfusions have remained of central interest to me and have dominated my medical and scientific life.

Life As an MD

After I passed my MD examinations in June 1952 (this time "with pleasure"), Professor Mulder offered me a residentship in internal medicine, starting the fall of that same year. I decided to earn some money in the meantime as a locum tenens. At that time the head porter of the hospital,

van Zon, a very important (and overweight) person, was in charge of giving such positions away. Luckily I got along well with him, and he arranged for me to take care of not one but two GP practices in a village near Leiden—Ter Aar. I spent almost half a year there, and it was one of the richest medical experiences of my life. In those days, as an MD without further specialization, one was allowed to do almost everything including small surgery and obstetrics. I got a taste of that and a lot more: doing housecalls on a small motor bike, which regularly either refused to work or drove me into one of the many canals; trying to establish my authority as "the" doctor, which was not easy because of my youthful appearance; diagnosing a chronic lymphoid leukemia which presented itself as a heart failure; finding two children under the bed on which a delivery took place; trying to control the hysterics of a captain of a touring motorboat who had collided with a bridge, and so on. I also got here my first taste of medical commercialism: I was called a nitwit because I had given a sample of a skin ointment to a (rich) farmer's daughter without charging her! I also made my first serious mistake by diagnosing too late a broncho-pneunomia in a farmer with a scleroderma, which killed him. Life could be grim.

On the 1st of November 1952 I reported to the Department of Internal Medicine. I got two white coats from the hospital, put my stethoscope in my pocket, and looking (I thought) much "the" doctor I went in search of room 7 where the blood bank was located. In those days it was customary for the most junior resident to be in charge of the blood bank. The blood bank, an initiative of Professor Mulder, turned out to be no more than a room with two couches, a refrigerator, a microscope with a mirror, and a centrifuge which worked on hand power. When I came in I saw two young ladies in serious discussion, who completely ignored my presence. I said "ahum"; they looked up, slightly irritated and one of them said: "Oh, that is the new doctor. You better sit down until we have some time." I felt deflated, sat down, and started to look at the papers on the desk. I noticed Mollison's monograph: "Blood Transfusion in Clinical Medicine." The ladies finished their business and introduced themselves: Suus Kloots and Aad van Leeuwen. Little did we expect that 40 years later Aad and I would be still working together! We have collaborated in almost all our scientific activities, and many of feats I describe here would in all probability not have taken place if we had not!

Aad and Suus explained to me the, at that time, very simple routine of the blood bank and warned me that I was "medically" responsible for all the blood transfusions given in the hospital. A few weeks before a mistake had been made. A hemolytic transfusion reaction had occurred, and my predecessor had been fired on the spot. This was a good incentive to get

acquainted with the administration of the blood bank. It paid off. I realized that the number of blood transfusions given had risen dramatically over the last half year and that if something did not change soon we would be short of donors in three months time. I made a graph illustrating the point and went to Mulder. He was busy and told me to get off and not to bother him. Precisely three months later we ran into the problems I had foreseen, and I went again (with my graph) to Mulder. His reaction was typical: "van Rood, you have a good head on your shoulders. I was wrong and you were right; you have 'carte blanche' to put things in order." And that was the way I became the Head of the blood bank for the rest of my career. I liked being in charge, and I was soon setting up the administration, reorganizing the medical inspection of the donors (we found two cases of open tuberculosis, a case of chronic lymphatic leukaemia, and many other serious pathologies), and above all improving the laboratory equipment and techniques.

The Central Laboratory of the Red Cross in Amsterdam under the guidance of Jochem van Loghem Jr. was our gold standard. I spent several months there under the tutelage of Mia van der Hart, his head technician. We introduced the anti-human gamma globulin technique, then called the Coombstest, for the detection of sensitization against rhesus and other bloodgroup systems and for cross-match purposes. Van Loghem organized quality control exercises; it turned out that almost half of the hospitals in Holland at that time (1954) were not able to do a decent cross-match or reliable bloodgroup typing.

In 1954 when Professor Gerard Brom set up a department for open heart surgery, the demand for blood tripled. We got a more than adequate increase of staff but no extra space and soon we were bleeding donors in the corridor and waiting room and doing cross-matches in the toilets. Leukocyte agglutination had become popular, so we had to do that too. We squatted in the stable where originally the horse was housed that had been used to make antidiphtheria serum. Probably that clinched it, and the hospital administration with only minimal priming from our side, promised an addition to the department all for us! It was ideally located, near the wards I was looking after, and it was 3000 square feet. We were delighted. For the opening we set up an exhibit explaining what a blood bank was all about. We then comprised a staff of over 16 people including George Eernisse MD, another lifelong partner and colleague. A few years later (and another expansion) Hans Bruning, a biochemist joined the team and set up Immunochemistry. He is still working in the department and virtually singlehanded has developed the automatic reading of HLA serotyping. But that was much later.

My longest residency was with Endocrinology under the supervision of

Querido. He was (and is) a remarkable intellect. He received much of his training in Paris and was a clinical scientist of the same class as Loeb. He lectured well but was at his best when he led the Thursday morning discussions in the outpatient clinic. His main interest was iodide deficiency and its sequelae, not only in the western world but also in Indonesia. He was however quite broad and for instance knew Doniach and Roitt quite well, especially for their work on autoimmune thyroid antibodies. For one of the grand rounds they analyzed the serum of a patient of Querido and sent in a beautiful precipitation curve. I was supposed to discuss the case, was badly prepared, and bungled the presentation. Querido did not talk to me for weeks. In general, however, we got along quite well.

Each year of my residency a group of the residents under the guidance of Querido and Goslings visited another University Hospital, inside or outside Holland. During such a visit to Paris, I first met Jean Hamburger, the nephrologist who would later play an important role in kidney transplantation. The visit has remained with photographic clarity in my memory: Hamburger, already then a quite impressive person, rushing in, quite "échaufé" because he was an hour late for our appointment. He got stuck after a car accident in which he was involved. It was also here that I saw anuria, after an illegal abortion attempt, in a beautiful young girl.

When we started to put a Department of Haematology together (until then nonexistent in Leiden), we took endocrinology as an example. We were myself, Loeliger, a Swiss MD, who had worked with Koller in Zürich and had identified Factor VII, Lopes Cardozo MD, pioneer in clinical cytology, and Leeksma MD, chef de clinique with Querido, who was writing a thesis on the survival of platelets labelled with DF32P.

We worked mainly as consultants, but we did have "our own" patients of which we took care if they were hospitalized. All this kept us quite busy and there was little time for research. Loeliger did best, producing beautiful curves depicting the kinetics of coagulation factors and writing quite important papers. I must confess that I was jealous—more of the graphs than the papers.

After an unsuccessful attempt to screen for hepatitis B (unpublished), we concentrated on survival time studies of platelets and erythrocytes, and correlated them with the presence of auto- and alloantibodies. In the beginning we used DF32P; 51Cr came available much later. Because of the short half life of DF32P we were forced to work without a break for two to three days after the labelled DFP had arrived. Sacha brought us food. When we could not keep our eyes open we took a nap on one of the couches for the blood transfusion donors. That work formed the basis for the thesis of George Eernisse and later Leo Bosch, and not only confirmed

that erythrocytes survive for 120 days and platelets for 10 days, but also analyzed the influence of auto- and alloantibodies on survival.

At that time we had become interested in alloantibodies not only against red cells but also against white cells. The main reason for the latter was that I had to take care of a patient, R., who came every month to our blood bank to receive a blood transfusion. During the war he had driven a lorry which ran on methane and possibly as a result he had developed a severe, full blown aplastic anaemia.

His visits to us were noteworthy, not just for his good spirits but because each blood transfusion induced a nonhemolytic transfusion reaction of the most severe kind with hypotension, nausea, and above all shaking chills. The hospital bed literally shook! He received antiallergic drugs and even cortisone to no avail. We sent the blood to the Central Laboratory of the Red Cross in Amsterdam, and they confirmed what we had found: the patient had strong agglutinins against the white cells of a panel of healthy donors. And that was important for our patient because van Loghem's group had just shown that these leukocyte agglutinins could cause severe nonhemolytic transfusion reactions and that this could be prevented simply by removing the leukocytes from the transfusate (5). Simply, that is, if you had the equipment to do so; we had not. To solve this problem I came to the hospital quite early in the morning when the nurses on night duty told the nurses on day duty before they took over, what had happened during the night and took from the cupboard two liver biopsy needles, which was strictly forbidden. The liver biopsy needles were at that time considered to be very expensive, and only the head resident was allowed to use them. With them and a suction pump I was able to remove the leukocytes, and R. had no more shaking chills. That was the situation in 1958. We had a reasonably well-equipped laboratory with people who had experience in serology and cell kinetics. We were accepted in and were part of the clinic where we had our own playground: immunohaematology, and we had been trained by the best people of that time in clinical research. Then it happened.

Enter: HLA

On April 14, 1958, Mrs. H., a mother of four children, was delivered of twins. Soon afterward she had postpartum bleeding that led to pre-shock and she was transfused. Her blood pressure rose again, but a few hours later the patient had shaking chills, felt nauseated, and collapsed. Thanks to Van Loghem's findings we realized that the patient probably had suffered from a nonhemolytic transfusion reaction caused by leuko-agglutinins. Strong leukoagglutinins were indeed found, but that in itself confronted us with a question: Where and when the patient had received

the blood transfusions? Blood transfusions were thought at that time to be the only inducers of leukocyte antibody formation (6, 7). A report had even been published that no antibodies had been found in 100 women with previous pregnancies. Leiden is a small town with only one blood bank, and the patient was born in Leiden and lived there, but George Eernisse could find in the blood bank no record of previous transfusions. When we went back to the patient to ask where she had received blood transfusions she looked at us in horror and said: "This was the first blood transfusion I have ever received, and as far as I am concerned, it will be the last."

Only then did we realize that although the reports concluded that leukocyte antibodies are only formed after blood transfusion, they might be wrong and that pregnancy per se could induce leukocyte agglutinins (8). For some still unknown reasons such agglutinins remain stable for many years after delivery. For that reason they are much better suited for immunogenetic studies than are the agglutinins induced after blood transfusion which tend to lose their specificity quickly. One of our best sera, produced by the wife of a butcher from a nearby village, was found 35 years after her last delivery. It was with such sera that the first family studies were carried out. The groups were given numbers. The serum detecting group 1 was the one from Mrs. H, who had opened our eyes. The sera for groups 2 and 3 were both oligospecific, a term coined by Walford to indicate that with cross-absorption experiments one could make a pretty good guess that there were only a few different specificities in a serum. These data were presented in Rome in 1958 during the hematology and blood transfusion meeting. In the same meeting Dausset presented his data on Mac, now HLA Az. The meeting was memorable for other reasons as well.

We were of course newcomers in the world of white cell serology, but we knew quite a few people in the blood banking world. One of them was Dr. James, head of the Northern Blood Transfusion Service, a gigantic operation. Dr. James as the director looked and acted the part. When we told him about our leukocyte antibodies induced by pregnancy and especially when we told him that these could be absorbed by platelets, he looked at us with a knowing smile and said: "Well, I don't know but you might have to eat your hat" (an expression we had not heard before, but it was quite clear that Dr. James did not believe us). This scepticism was general at the time. That same morning we met Dausset and Colombani on the steps leading to the congress building and they were arguing about the same point: Are platelets able to absorb leucoagglutinins. When Dausset heard that we had found the same, he turned to Colombani and said: "What did I say! Are you now convinced?"

One could tell many similar anecdotes, all reflecting how little we knew

and how uncertain we were about our findings. This was certainly, in no small measure, due to the poor reproducibility of our techniques: 70% for the defibrinated agglutination technique most people were using, and 80% if one used EDTA as an anticoagulant, a technique introduced by Wasastjerna and popularized by Van Loghem and his coworkers, especially Engelfriet.

Another technical improvement was the finding that certain leukocytes samples, although they were not agglutinated by a given serum, sometimes were able to absorb the antibody out of the serum. This was called the ANAP phenomenon (agglutination negative, absorption positive), later also called CYNAP for the cytotoxicity assays. The first serum so studied, nr. 36, turned out to be the first of a cluster of sera which recognized the same antigen. We called it Antigen Four [now HLA-Bw4].

Next we set out to do what was at that time considered a very large experiment: 60 sera obtained from women who had been pregnant; the sera contained leukocyte agglutinins that were tested against the leukocytes obtained from a hundred random panel donors. This is now a standard procedure which still forms an essential part of all workshops, but at that time (1960, 1961) something like this had never been done. It took several weeks to do all the tests (Figure 1). The only way we could analyze the data was by comparing positive and negative reactions obtained by each serum against the positive and negative reactions of all other sera and then doing two-by-two chi square tests. The idea to do it in this way came to me when I followed a course on medical statistics. I realized that if two sera were tested against the same panel of leukocyte samples (even if their reaction pattern was not completely identical) and if they showed a significant association, this meant that they must have "something" in common and that that "something" could be an antigen they both recognized. All rather obvious now, but at that time it was new, at least for us. Doing such an analysis by hand (i.e. 1770 comparisons) would be, if not next to impossible, quite boring. We decided to find a computer to do the work for us. The only computer Leiden University owned was located in the Department of Astronomy and was used for the calculations of distances, speeds, etc. It was completely useless for the kind of analysis we wanted to do. By chance we heard about a Czech firm in Utrecht and went there. They had in a big attic an enormous "cuckoo-clock," a mechanical Hollerith sorting system, all wooden hammers and wheels. They were very nice people who really understood what we were talking about. A week later they came proudly with the analysis. It was fantastic; they had understood what we asked for. The only thing wrong was that they had compared not every serum with every serum, but every cell with every cell! Quite a number of very interesting patterns came out, but it was too early

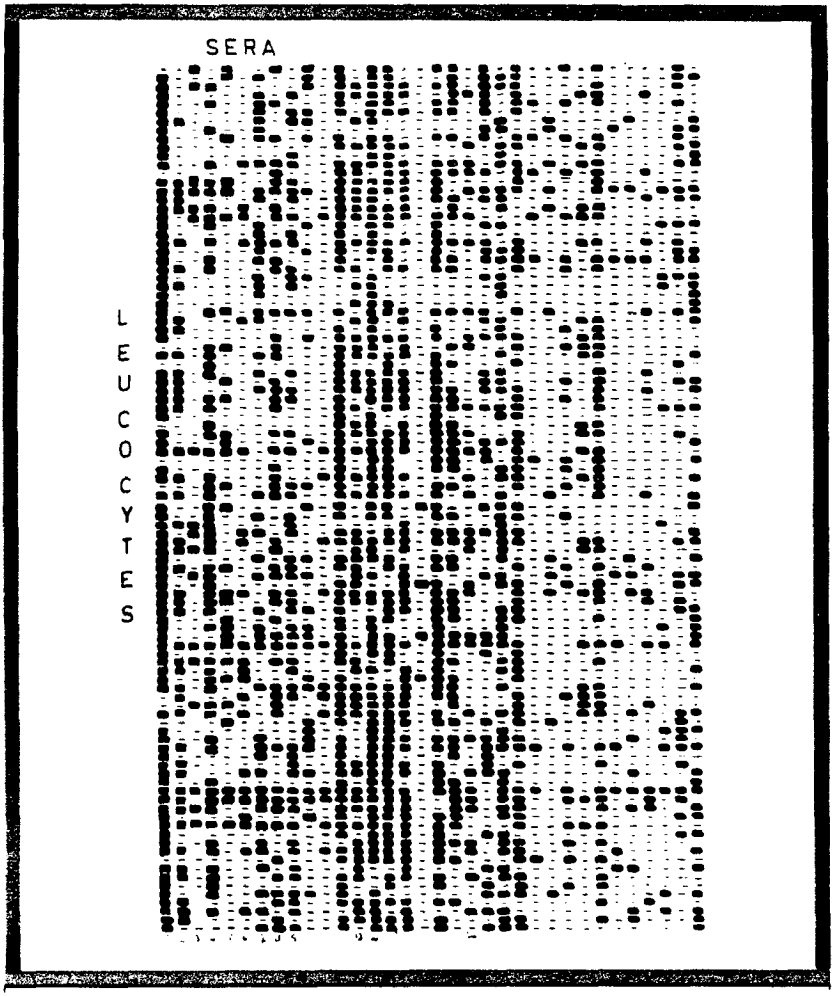

Figure 1 Agglutination pattern of 34 sera with leukocyte antibodies tested against 100 leukocyte samples. Every vertical row of squares and hyphens shows the results obtained with one serum against the panel; every horizontal row shows the results of the 34 sera with one leukocyte sample. Black squares mean agglutination positive; hyphen means agglutination negative. Doubtful results were recorded as agglutination negative (9, 10).

for us to interpret them. After that they went bankrupt, and we have never known whether this was because they had done these experiments for us or for some other reason. I must confess we did feel a bit guilty.

Anyhow, the search went on. The Bureau of National Statistics was able to do the job, and together with the department that was responsible for calculating the salaries of the Dutch government's employees, we got our first really good analysis. The pattern that came out is shown in Figure 2: two very nice clusters which we named 4a and 4b, HLA-Bw4 and -Bw6 now. All this happened in 1961 and we presented the work for the first time in Vienna during the meeting of the European Society of Haematology. Our studies were put together in 1962 in a thesis: "Leucocyte Grouping, a Method and its Application," a title suggested to me by my friend and colleague Fred Loeliger, the coagulation expert. In Holland a thesis is a real printed book, and we proudly sent it to all the workers in the field (9, 10).

After I had defended my thesis successfully, Querido insisted that I take a sabbatical. At first I was less than enthusiastic about this. Leukocyte typing had become quite an important aspect of my life; it was clear that it could be of importance in the clinic, certainly for platelet transfusions in hyperimmunized patients and possibly for transplantation. Querido was insistent, and for that I am still grateful to him.

The next question was where to go. I consulted Jeanette Thorbecke, then already at NYU, and she arranged that I could spend a year with Frank Adler and Marvin Fishman, then at the Public Health Research Institute of the city of New York. The institute had some excellent people such as Hirsch, then the chairman, and there was some very good virology, but their main thrust was in the direction of nucleic acid research. Adler came originally from Vienna and was one of the, even at that time, rare persons who oversaw the whole field of immunology. He took it on himself to initiate me in the field by spending about one hour every day taking me through Kabat and Meyer's textbook, chapter by chapter. A unique experience. He had a laboratory overlooking the East River. I was sitting across the hall in a room where Freund had some decades before concocted his famous "adjuvant." Marvin Fishman worked with Adler and had attained recognition by describing the phenomenon that extracts from primed lymphnodes containing RNA were able to induce an antiphage antibody response in virgin rats. His results were later thought to be caused by carry over of antigen. Marvin was New York born, highly intelligent, and very nice. He trained me in many of the basic techniques, including working with rats.

The Institute had close connections with NYU, notably with the depart-

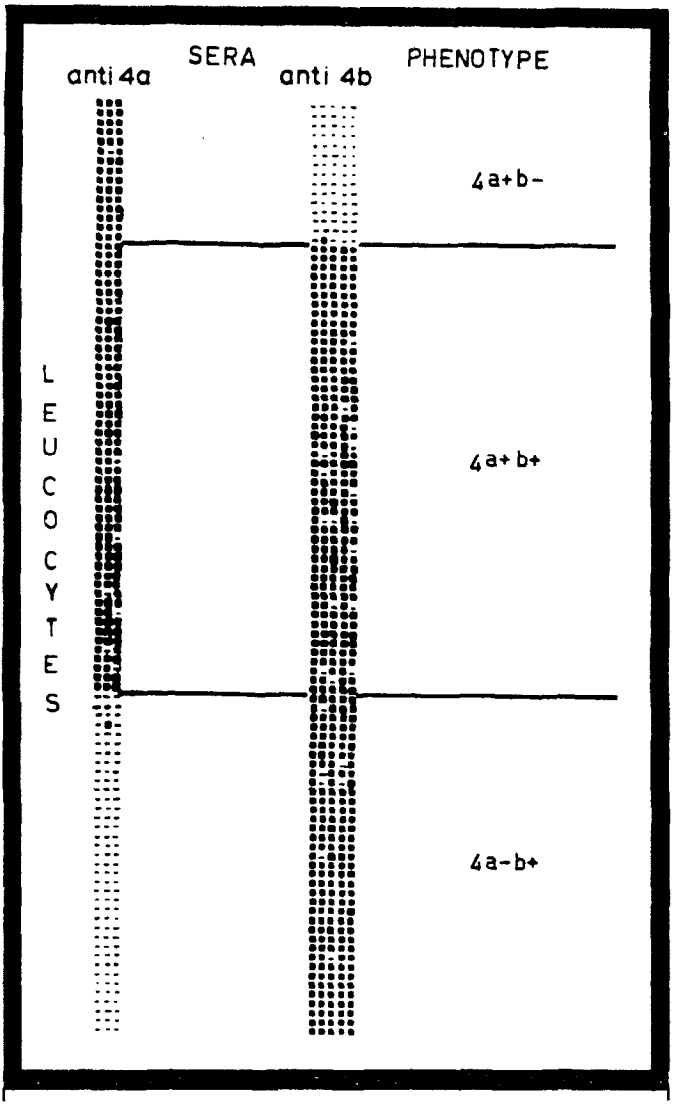

Figure 2 Agglutination pattern of 8 sera with the leukocytes of the panel. Every vertical row of squares and hyphen shows the results obtained with one serum against the panel; every horizontal row shows the results of the 8 sera with one leukocyte sample. A black square means agglutination positive; hyphen means agglutination negative. Doubtful results were recorded as agglutination negative (J. J. van Rood 1961)

ment of Immunology with Baruj Benacerraf, Zoltan Ovary, Jonathan Uhr, Ellen Vitetta, Jeanette Thorbecke and many others. We met every Tuesday at lunch for a journal club. On request of Benacerraf I lectured for the students on transfusion medicine.

New York was (and still is) a fascinating place: the seminars at Rockefeller, the Saturday morning seminars at Columbia University on nucleic acid research, but also the city itself, the museums, the music, the river. We had several family members in and around the city which made it easier to get a taste of American life, a fantastic experience.

I met here also for the first time many of my lifelong friends: Peter Elsbach, a Dutchman also working at NYU on bactericidal substances from leukocytes, and Pablo Rubinstein, a Chilean immunohematologist (whom I met for the first time in Fred Rosenburg's Blood Bank in Mount Sinai Hospital), highly intelligent, technically very gifted, and far too modest. I visited him in Chili and he came to Leiden for several small sabbaticals. Many other encounters could be mentioned, such as the day that I was talking to Kurt Hirschhorn in his room in NYU and a small darkhaired student sitting behind a microscope interrupted our academic discussion several times by patting himself on the shoulder and exclaiming: "Look what a beautiful blast! Wow, I did a real fantastic job!" It was Fritz Bach! We have remained close friends ever since.

After my sabbatical in New York, Sacha, our two and a half children, and I went on a trip through the States. We visited the Niagara Falls, Old Faithful, Glacier Park, Death Valley (where we almost lost our son because of heatstroke), Yosemite Park, and many other beautiful sites. We also visited Schilling who had developed his vitamin B12 uptake test, Bob Good who told me that they too had discovered the leukocyte antigens, Don Thomas still working on dogs, and Rose Payne who introduced me to Herb Perkins and Walter Bodmer. Querido had been right: My sabbatical not only introduced me to immunology, it also opened the (scientific) world to me.

Rose Payne and Walter Bodmer were the first to introduce our computer approach in their laboratory. A few months later I got a letter from Rose Payne saying: "Dear Jon, Could you send us another copy of your thesis. The copy you sent to me has travelled so often between Walter's lab and mine that it is completely falling to pieces and we would like to have a new one." We sent it immediately. In 1964 they described the LA1, LA2, and LA3 antigens (11). It was (and still is) of interest that using the defibrinated leukoagglutination technique, they identified the LA or HLA-A locus antigens, and later the HLA-B locus antigens while we, using the EDTA

leucoagglutination technique, identified the Four or HLA-B locus antigens only, (with the exception of HLA-A2, which was recognized as well). Might CD8 have something to do with this (12)?

In that same period two important technical improvements were realized. The first was the introduction of the complement dependent cytotoxicity assay by Batchelor and others, and the second was the development of the microcytotoxicity test by Terasaki, a truly revolutionary technical improvement. If you were very good you could do 20–25 agglutination tests with one ml of serum, but with Terasaki's microcytotoxicity test you could do a thousand cytotoxicity tests (13)!

During my sabbatical Bernard Amos together with Ruggero Ceppellini had visited Leiden where Aad van Leeuwen, after consulting me, showed them everything we had done so far. After that visit Bernard decided to organize the first histocompatibility workshop. This was one of the significant initiatives that led to the rapid development of the HLA field during this time.

The first meeting in 1964 was at the National Academy in Washington; the workshop was in Duke University, North Carolina. It centered mainly around a comparison of techniques. I was asked to summarize the meeting, a hopeless task because everybody had used different cells, different sera, and different techniques. The only thing I could think of was to invite everybody to Leiden where we had a panel typed for seven well-defined antigens. The 1965 workshop thus set the format that was followed during all subsequent workshops: a panel of cells was tested by the different teams using their own sera and techniques.

The results were most gratifying, and it was decided to continue the workshops. The 1965 workshop in Leiden was also a turning point because during this meeting family data from our group and population data from Dausset provided preliminary evidence that most of the antigens recognized belonged to one genetic system. At the time that was revolutionary. The idea that there could be one major histocompatibility complex did not exist. After all, our thinking had been strongly influenced by the discovery of the many blood group systems that were mainly genetically independent, while most HLA workers of that time knew little or nothing of H2. It came as no surprise when Amos stated (in the discussion) "Probably quite a few people would like to take issue with you on the evidence that this is one system . . ." (14). Amos was quite right. We had observed similar associations but had also shown that some of the antigen clusters that had significant associations between them included sera, which recognized the Group Five system. It had already been shown that the Group Five system was independent of the other antigens. We had called that one genetic system the: Group Four system, Payne and Bodmer

called it: LA and Dausset: Hu. After an emotional meeting in Williamsburg we dropped Group Four, and HLA was born.

HLA Coming of Age

The 1965 workshop was also memorable for the demonstration that the HLA antigens are transplantation antigens. The protocol was simple. Peter Medawar had shown in his classical experiment that if a piece of skin was exchanged between two rabbits, the transplant was rejected after 10 days, but when a second piece of skin of the same donor was transplanted to the same recipient, that piece of skin was rejected in 5 days. Medawar concluded that the first skin had immunized the rabbit and that homograft sensitivity had been induced. At the time this was a really new finding. In the next experiment Medawar did not exchange a piece of skin but instead injected the buffy coat cells of a blood sample from the donor intradermally in the recipient, and only after that transplanted a piece of skin from the buffycoat donor. This first transplant was rejected not after 10 days but after 5 days; in other words an intradermal injection of leukocytes could induce the same homograft sensitivity as a skin transplant. It follows from this that these leukocytes must carry the same transplantation antigens as skin. With this the whole field of transplantation opened.

All that remained to be done was first to recognize on leukocytes those transplantation antigens, e.g. by serological methods, and second to show that matching for these antigens improved graft survival. The first task we had done, and the second was possible, thanks to Felix Rapaport who had developed a very nice skin grafting technique (15). We of course needed volunteers. We approached many of the colleagues who had helped us with building the big panel by which 4a and 4b had been identified; they got the shock of their life when we asked whether they would be willing to give and/or to receive a skin graft. George Eernisse and I set the example, and they agreed to collaborate—this was long before the days of medical ethics committees. Following a protocol we had worked out with Balner in monkeys, and which I had presented in Stockholm in the summer of 1964 (when I met Erna Möller for the first time), we intradermally injected 2×10^8 leukocytes incompatible for one antigen only (B7) with the recipient, and then two weeks later we transplanted a skin graft of two unrelated donors of which one was B7 negative and one B7 positive. Mean skin graft survival was 11 days for the B7 negative donors and 6 days to the B7 positive donors, proving that HLA antigens were transplantation antigens.

After the Leiden workshop we travelled to Torino where Ruggero Ceppellini and his group had exchanged skin transplants between family members to perform the HLA typing of donors and recipients. We could indeed

show that in nonimmunized recipients graft survival correlated with HLA matching. Matching for the independent Group Five system apparently had no influence. Amos made a similar observation at almost the same time.

In 1966 flying back from Zurich to London I discussed our findings with Sir Michael Woodruff, the discoverer of antilymphocyte globulin (ALG). Woodruff became quite enthusiastic and persuaded us to come to Edinburgh to study his patients and so we did. After Edinburgh we set out with Aad van Leeuwen, Ali Schippers, and Hans Bruning on an Odyssey around the transplant centers of the world. We had to be on the spot to be able to do the leukocyte typing because we used an agglutination assay and the cells had to be fresh. In this way we were able to collect data of over a hundred patients and their donors. When we analyzed the sib transplants, we found that we had far more HLA identical siblings pairs and far fewer incompatible ones than expected. The 18 patients we were able to type came from a group of 40 of which 22 had in the meantime died. At that time, if chronic dialysis was not readily available and if a patient rejected a transplanted kidney this meant certain death. The conclusion was clear: when donor and recipient are HLA identical it is less likely that the patient will reject the kidney, and for that reason he/she remains alive. We had proven with this study (and in a similar study in which the donor was the parent) that the antigens we were able to recognize by serology on leukocytes were transplantation antigens important not only for skin graft survival but also for kidney graft survival. We were very excited.

Shortly after the completion of this study the Third Histocompatibility Workshop took place in Turin, where Terasaki and Dausset presented similar data (1967). It was at that meeting that the proposal was put forward to set up an international organization for organ exchange: Eurotransplant (16). The name Eurotransplant had come to me when I was passing a big truck on which was written EUROTRANSPORT. Changing only three letters the name Eurotransplant came into existence.

The proposal to start Eurotransplant got in the beginning a rather mixed reception. I must say that with one or two exceptions, the colleagues closest to me were rather sceptical. Nevertheless the idea received the support of quite a few transplant centers, in the beginning especially in Belgium. The first kidney was flown in by helicopter from Louvain and transplanted in September 1967, much earlier than we had planned. In 1967 a total of 11 kidneys were transplanted under the auspices of Eurotransplant. In 1968 this number rose to 60 and then it steadily increased. This was a good time. In a close cooperation between surgeons, nephrologists, and immunologists, each patient was discussed before being selected for transplantation, and the whole team was activated when a kidney became

available. I remember quite well the direct involvement with the transplant procedure and how I enjoyed it.

The first analysis of the Eurotransplant data was presented at a meeting of the International Transplantation Society in The Hague in 1970. The meeting became quite famous, not so much for its scientific importance as for the very liberal availability of Dutch beer and gin and the excellent social program. I was supposed to give an overview of the activities of Eurotransplant at that meeting. However, when we analyzed our data we found that there was really very little to be said about the effect of matching. One thing we noted though was that our results were much better than those reported by the International Registry for Kidney Transplantation. At the time we assumed that this was due to our better than average matching, but we had no hard data to support this. With a longer follow-up however, it became clear that in these first years we had indeed improved long-term graft survival. The discussion whether HLA matching improves organ graft (and patient!) survival has remained with us until this day, although it will of course be missed if HLA typing of donor and/or recipient are not faultless.

The contributions of the workshops were many, and we can mention only a few highlights. The first is Ruggero Ceppellini, who (together with Walter Bodmer) gets the credit for educating the HLA community in basic genetics. Ceppellini has made a number of very important contributions in the field of immunogenetics, and to science in general, including immunology. He has been in many ways the brain behind many of our experiments. He also had a very strong personality.

For the Torino workshop family studies were to be the focus. Ceppellini suggested that it would be fun to have some duplicates, and he set out to select families with monozygotic twins. When the data were analyzed, it turned out that with some of the techniques, especially the very sensitive ones, it was impossible to differentiate between monozygotic twins and dizygotic twins.

Ceppellini with Mattiuz, Piazza, and Bodmer was the one who introduced the concept of linkage disequilibrium in the HLA field. He was also instrumental with Miggiano and Lightbody in introducing the CML test, the cell mediated lympholysis test. When Lightbody presented the data for the first time in Paris and was asked why he called this test the CML test, he answered: "Well, officially we say it is the cell mediated lympholysis test, but you of course understand that it stands for: Ceppellini, Miggiano, and Lightbody." (17) .

There are at least three other events I would like to mention. One is the birth of the MLC test. Again, many stories prevail. Fritz Bach, whom we had met while visiting Kurt Hirschhorn and Barbara Bain, discovered

independently at about the same time the MLC test. Bach overheard a discussion between Jim Gowans and Peter Medawar, in which they reflected on the fact that lymphocytes appear to carry transplantation antigens because they could induce homograft sensitivity, and that they could also form blasts and could divide. Fritz thought: "Well, if lymphocytes can do that, why not mix cells of two individuals and see what happens." The two-way MLC test was born, later to be refined by Ceppellini to the one-way MLC test (18). Barbara Bain stumbled on the MLC test in a serendipitous fashion. By mistake cells from two individuals were mixed in a tube, and although the PHA had been forgotten, blast formation was observed: Barbara Bain realized what she had found (19).

The MLC test made it possible to type for HLA-D. Bradley (20) was the first to do HLA-D typing in pigs, using homozygous typing cells [HTC]. Unaware of Bradley's paper we reinvented the wheel and were looking for homozygous typing cells to be used for HLA-D typing. We realized that they could best be found in the offspring of cousin marriages. The problem obviously was how to identify such people. Fortunately, we were aware of the fact that the Catholic church does not allow cousin marriages unless the Pope had given permission, and of course the Catholic church keeps excellent archives on all matters including these cousin marriages. We wrote a letter to the Pope asking for his permission to use that information. Sure enough after a few months we received a letter back that we should contact the bishop of Haarlem. We went there to discuss the problem, got his blessing, and went ahead.

Dausset heard the story and said: "What can be done in Holland should certainly be possible in France." He went to the Evèque de Paris and explained his case, but the Evèque de Paris said: "Non" and that was the end of it! To solve the problem Degos and Colombani went to the Touaregs who must marry their cousins. Unfortunately (and interestingly) they found far fewer homozygous children than expected. Was this due to chance, lethal genes (W. Bodmer) or diminished resistance against infections? All possible, but none proven.

After Torino, the Los Angeles (1970) and Evian (1972) workshops took place, reinforcing the importance of HLA for organ transplantation and providing a first attempt at making an HLA map of the world. In Evian, Eijsvoogel presented his elegant data on a family with a crossover between HLA-D and HLA-B and another between HLA-B and HLA-A. He could show that one needed both an HLA-D and a class I disparity to get a positive CML test (21).

While the recognition of the HLA-A and -B antigens has been systematically worked out since 1962, it took another ten years and a partly new methodology to attain the same for HLA-DR. This was because it

took several years to recognize that the determinants which stimulated in the MLC test were not identical to HLA-A and -B (as originally assumed), but also because even more time was needed before the idea was accepted that the HLA-D locus was polymorphic like the HLA-A and -B loci and also before methods to recognize this polymorphism became available. As a matter of fact the identification of HLA-D and of -DR took place simultaneously.

Much of the pressure to search for methods to type for HLA-D and especially -DR came from those involved in clinical organ transplantation. It had already been recognized that low or negative MLC reactivity improved graft survival of both skin and kidneys in humans and monkeys. Although typing with homozygous typing cells made typing for HLA-D possible, it was too time consuming to be useful in clinical renal transplantation using cadaveric donors. A serological method would be the solution if such a method could be developed. There existed in the beginning considerable scepticism that this would be feasible. Much of this scepticism was based on the fact that in many other systems T cell–dependent immunity differed in its specificity and antigenic requirements from B cell dependent immunity.

That we decided to study the problem of HLA-D related serology anyhow was in fact due to two observations, one of which was made by Ceppellini and his coworkers and one by us. Ceppellini had shown that sera with anti-HLA-A and -B antibodies were able to inhibit the MLC test. Although MLC inhibition by HLA antibodies was thus a well-established phenomenon, the mechanism by which this occurred was not understood. Again serendipity opened our eyes. A woman, mother of two children, had received an MLC positive but HLA-A and -B identical experimental skin graft. On the basis of previous experience we had expected it to survive 15 days at most, but at 23 days it was still not rejected. We wondered whether there might be "something" (an antibody induced by pregnancy?) that neutralized the rejection-inducing effect of an HLA-D mismatch.

We decided to use the MLC inhibition technique to screen for anti-HLA-D (now DR) antibodies. From the beginning it was clear that the test system had to be rigorously controlled for us to be certain that irrelevant antibodies (i.e. other than anti HLA-DR) could not cause MLC inhibition. To attain this we used responder cells obtained from a person, in most cases a woman with a history of multiple pregnancies, who had formed leukocyte antibodies. To exclude interference with anti-HLA-A and -B antibodies, stimulator cells were used that were HLA-A and -B identical with the responder cells. We named this approach the MLC inhibition test using SD identical stimulator cells or MISIS for short. The sera tested contained strong anti-HLA-A and/or -B antibodies. The very

first serum tested in the MISIS test was informative (Table 1) (22). Some responder-stimulator cell combinations were inhibited (P, N, T, U, V, W) while others were not (Q, R, S). This finding was reproducible, and other sera that showed similar results were found easily.

More direct evidence that antibody mediated the MLC inhibition was obtained when Aad van Leeuwen together with Riek Schuit developed a sandwich immunofluorescence test with anti-IgG which gave an at that time unusually low background with normal lymphocytes. While the background was 4–8%, some of the stimulator cells when tested with the serum used in the MISIS test showed bright staining of 16–17% of the cells (Table 1). These very important results could be obtained because Aad van Leeuwen had spent (as usual) many long evening hours counting all individual samples. The low background immunofluorescence was clearly essential to detect these small differences and was originally dependent on very rare and very pure rabbit anti-human Ig sera which had been made by Jiri Ràdl in the Laboratory of Experimental Gerontology-TNO, in Rijswijk. When it was understood that the background staining was due to binding of fluorescein labelled immunoglobulins by Fc receptor molecules, commercially available sera were subjected to pepsine treatment to remove the Fc part of the anti-human Ig, and the test became generally available. As is illustrated in Table 1 the first serum to detect polymorphism in the MISIS test also showed significantly positive fluorescence. The results of the two tests correlated quite well, a finding substantiated in later studies.

Table 1 Typing for MLC LD: MLC inhibition and immunofluorescence

Parous Woman Sch: HL-A2,3,7, W10				
	AB Serum (c.p.m.)	Serum Sch (c.p.m.)	AB Serum/ Serum Sch	Fluorescent (% Pos. Cells)
Sch + P_m	3,300	400	*7.3*	*17*
Q_m	14,000	8,000	1.7	4
N_m	9,700	1,100	*9.5*	*17*
R_m	10,000	6,400	1.6	9
S_m	4,200	2,800	1.7	7
T_m	5,200	700	*7.4*	7
U_m	13,500	1,300	*10.0*	*16*
V_m	26,500	1,100	*24.1*	*17*
W_m	1,800	200	*10.0*	–

All stimulator cells were SD-identical with the responder cells Sch: HL-A2,3,7,W10. The column AB Serum/Serum Sch depicts the inhibition index values obtained by dividing the c.p.m. obtained by culturing the cells in AB serum by the c.p.m. obtained when they were cultured in serum Sch. The significant inhibitions are in italics.

From A. van Leeuwen et al 1973, ref. 22.

It was concluded that the MLC inhibiting substance and the antibody that reacted in the immunofluorescence test were probably the same IgG molecule. Together with Bob Winchester we showed that many of the sera contained complement-dependent cytotoxic antibodies that reacted with B cells, and monocytes but not with (most) T cells and all platelets. The test was standardized and with minor alterations already used during the 6th and more extensively during the 7th Histocompatibility Workshop.

Although the rosetting B cell test is quite adequate when investigating healthy individuals, the B cell enrichment is tedious and with the blood of patients suffering from some diseases quite difficult. To circumvent this obstacle a further refinement was introduced. Instead of enriching the B cells in the test cell suspension, they were identified with FITC labelled sheep anti-human Ig. The monocytes stained as well. Next a standard two-stage complement-dependent lymphocytoxicity test was carried out, and the dead cells were identified by ethidium bromide. The red fluorescence of the latter stain and the green of the first can be read simultaneously by appropriate selection of (blocking) filters. We called the technique the Two Colour Fluorescence test and published it (23). The publication was the cause of the only harsh words Pablo Rubinstein ever used to me: He had just published a technique which he had also called (rightly) a two color test: red for dead and green for live cells. I apologized and was forgiven.

With this the two requirements for the detection of the HLA-DR antigens had been fulfilled: adequate techniques and reasonably pure antibodies were available. The genetics of the system also offered little problem, because it was assumed from the beginning that HLA-D and -DR were closely related if not identical to each other (DR stands for D related, a point decided after the 7th Workshop by Ruggero Ceppellini, Walter Bodmer, and myself).

While the techniques were developed for HLA-DR typing, the study of HLA-D using homozygous typing cells had progressed quite rapidly. Our group was especially fortunate because Keuning, Termijtelen, and co-workers had typed a well-sized panel and a number of families for HLA-D with homozygous typing cells, which facilitated the comparison of the distribution of HLA-D versus HLA-DR. The results showed that in the Dutch Caucasian population the distribution of HLA-D and -DR was very similar. We are writing now of 1975, the year of the Aarhus Workshop hosted by Flemming Kissmeyer-Nielsen, an old friend. Flemming has been in many ways an important figure and contributor to the HLA field in general and to our group in particular. He was the first to identify and recognize as such a crossover between HLA- A and -B, and he helped us set up the microcytotoxicity technique. Using that technique was a cornerstone of the whole Eurotransplant organization: the cells could be

easily shipped and stored and of course a thousand tests per ml is good mileage. He and Erik Thorsby provided many of the sera used in Eurotransplant. At the tenth anniversary of Eurotransplant I wrote them a note thanking them for their help while noticing that "many of our organ exchanges would have been blind dates" without them!

The Diversification of HLA

Probably the first patient whose life was saved by HLA was a woman admitted shortly after my sabbatical in 1964. She was suffering from a severe chloramphenical-induced aplastic anemia and was bleeding from all orifices. Platelet transfusions were at first successful, but when she formed leukocyte antibodies, she became refractory. Fortunately she came from a large family. We typed her brothers and sisters for the ten antigens we could then recognize [three of which did not belong to HLA], found some compatible family members, which we thromboferesed, also a novelty, in 1964. We got her through her aplastic phase and she recovered (24).

Shortly afterwards I went back to New York to attend a symposium on Immunohaematology at NYU, where I presented the case. Ray Schulman whom I had visited in NIH during my sabbatical and whom I admired for his beautiful work on delineating with the complement fixation technique the first platelet antigens (which later turned out to be in part HLA antigens) was present as well and afterwards he said: "Nice story, Jon, but I do not believe a word of it. It simply cannot be true!" However, it was and was soon confirmed by Yankee (25) and later reproduced by Anneke Brand and George Eernisse.

The role of HLA in kidney transplantation and the start of Eurotransplant in 1967 I have already mentioned. The importance of HLA matching in kidney transplantation is debated up to this day, although it is now generally accepted that if the HLA typing is good a significant matching effect can be found. The importance of HLA matching is, however, only relative in that many fully HLA-mismatched kidneys can function and function well for many years.

In bone marrow transplantation the situation is much more black and white: you need an (almost) completely HLA matched donor for a successful transplant. After a few attempts which failed, the first successful bone marrow transplant in Europe was performed in the Department of Pediatrics at the Leiden University Hospital in December 1968 (26), shortly after the first successful transplant in the United States carried out in Minneapolis by Bob Good and his team (27).

In those days we were strongly influenced by the group of Dick van

Bekkum who in beautiful systematic preclinical work, first in mice and later in monkeys, had shown that bone marrow transplantation could work. It was soon realized that only one out of three patients had a suitable HLA identical family bone marrow or platelet donor and that we needed a panel of unrelated HLA typed blood and bone marrow donors. We started with 150 students from a seminary in nearby Warmond but soon switched to blood transfusion donors. In 1970 we formalized this effort and called it Europdonor, in the hope that the other European HLA laboratories would join (28). This happened to a much more limited extent than had been the case for Eurotransplant, but it functioned well right from the beginning for HLA matched platelet donations and in the last five years also for bone marrow donations.

Ceppellini had already in 1967 stated that: "Nature had certainly not maintained a polymorphism such as that of HLA, only to frustrate transplant surgeons." True enough, while the HLA workers were discovering HLA and were getting to grips with its clinical importance, those who were working with animals laid the foundation for an understanding of its function.

In 1963, during my sabbatical in NYU I heard in an elevator Benacerraf tell someone vaguely familiar [Lewis Thomas?] that he had found that one strain of guinea pigs did respond to one of Michael Sela's antigens and another strain did not. This was the discovery of the Ir gene function of the MHC, although Benacerraf did not know at that time they were linked to the MHC (29). In 1964 Lilly, Boyd, and Old showed that susceptibility to Gross Leukemia Virus was H2 linked, and a year later Hugh McDevitt and Michael Sela showed the same for the antibody response against artificial antigens (30).

Soon the HLA community followed these leads, and inspired by Lilly's findings, Amiel presented during the Torino workshop in 1967 data which indicated that the occurrence of M. Hodgkin was HLA linked. The audience remained rather sceptical however. This was probably partly caused by Amiel's performance during the wet part of the workshop. Amiel's technician was ill and Amiel, who had very poor eyesight, read the results himself; they came out almost at random. Several years later Peter Morris confirmed his findings, however; glutenenteropathy followed, and when the link between ankylosing spondylitis and B27 was found, the excitement was general. Predictions were made about preventive medicine, diagnosis, and treatment on the basis of HLA typing—predictions which have still to be fulfilled. HLA disease association studies have received enormous attention; epidemiological studies on the importance of HLA in infections (probably its main biological function) are relatively rare, however. During the Evian Meeting in 1972 Piazza, Ceppellini and their colleagues presented

a classic study on the association between endemic malaria and HLA in four Sardinian villages, a study which has recently been beautifully confirmed and extended by Hill and colleagues (31,32). In 1976 Rene de Vries did the same for leprosy, then much debated, and in 1979 for an epidemic of typhoid and yellow fever among Surinam immigrants (33).

The description of MHC restriction by Zinkernagel, Doherty, and Shearer in 1974 was another milestone in our understanding of how the MHC works on the molecular level (34). MHC biology got its molecular basis in the work of Stan Nathenson and, especially for HLA, of Jack Strominger. Together with Hidde Ploegh, Peter Parham, Henry Orr, and many others, Strominger determined the amino-acid sequence of HLA-A2 and -B7 and cloned the gene (35–37). From that, DNA technology developed, and many considered this the end of the serology period of MHC. Jan Klein wrote: "It was fun while it lasted, but now it is over."

Looking back over the years and my involvement in HLA two factors have clearly played an essential role for me in my work: serendipidity was one and the double life I led switching back and forth between the clinic and the laboratory was the other. About the first I have said enough and the second should be clear from my story. Clues found in the clinic (a transfusion reaction, a skin graft that survived longer than expected) could be studied in the laboratory, understood, and then applied in the clinic. This was most gratifying, even when such work led to an involvement in the management of organizations such as Eurotransplant, which by the way can be fun.

The clinic, with or without the help of serendipity, continues to give clues that can lead to exciting new avenues of research. We are now in the middle of one: the finding that about half of the patients hyperimmunized against HLA have nevertheless not formed antibodies against the non-inherited maternal antigens (NIMA) (38). On the level of the T cell repertoire this can be found as well. The NIMAs may influence graft survival and susceptibility for rheumatoid arthritis and diabetes, but all of this is still in a research phase and does not (yet) belong in a preparatory chapter. I bring it up not only because it fascinates me but also to make the point that the clinic continues to give clues and that following these up can be fun.

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DIFFERENTIAL REGULATION OF MURINE T LYMPHOCYTE SUBSETS

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Abstract

Signaling pathways in T lymphocytes have been incompletely characterized. It is evident that differences exist among the T cell subsets. We have defined several distinct mechanisms that affect differentially the activities of murine T lymphocyte clones representing various $CD4^+$ and $CD8^+$ subsets: Interferon-γ (IFN-γ) inhibits proliferation of but not lymphokine production by T_H2 cells. IL–10 inhibits antigen-presenting cell (APC)–induced lymphokine production by T_H1 cells but not by T_H2 cells. Murine T_H1 and T_H2 clones proliferate optimally in response to distinct APC populations. T_H1 and T_H2 clones utilize different TCR-associated signaling pathways. High concentrations of antigen (or anti-TCR mAb) inhibit IL–2–induced proliferation (but not lymphokine production) by T_H1 and cytolytic T lymphocyte (CTL) clones only. Exposure of T_H1 clones (but not T_H2 clones or $CD8^+$ CTL clones) to IL–2 induces unresponsiveness to antigen. T_H1 and T_H2 clones as well as $CD8^+$ clones can be cytolytic, but not all T cells use the same cytolytic mechanisms. $CD4^+$ clones from some mouse strains are not cytolytic if they do not secrete IFN-γ. Understanding the mechanisms that differentially regulate the various kinds of T cells, in addition to providing insights into the molecular events associated with activation of those subsets, should facilitate modulation of their activities in vivo, making it possible to influence favorably the outcome of disease processes.

INTRODUCTION

T lymphocytes play a central role in immune responses, carrying out a number of effector and regulatory functions. While some of these functions

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are mediated directly through intercellular contact, many of them are carried out by secreted lymphokines. Thus, the characteristics of specific immune responses are determined to a large extent by the particular array of lymphokines secreted by T lymphocytes that participate in those responses. T lymphocytes have been divided into two groups based on expression of CD4 and CD8 cell surface molecules. Expression of these molecules is acquired during development in the thymus. Most T lymphocytes in peripheral lymphoid tissues can be divided into two subsets, one expressing CD4 and the other expressing CD8. This distinction generally but imperfectly correlates with fundamental functional differences: $CD4^+$ T lymphocytes carry out "helper" function and thus have been designated helper T lymphocytes (HTL), while $CD8^+$ T cells are cytotoxic to antigen-bearing target cells and thus have been designated cytolytic T lymphocytes (CTL). Expression of these cell surface molecules correlates better with the class of major histocompatibility complex (MHC) molecules that restricts antigen recognition. The response of $CD4^+$ T cells usually is restricted by class-II MHC molecules while the response of $CD8^+$ T cells usually is restricted by class-I MHC antigens. $CD4^+$ HTL appear to exert most of their functions through secreted lymphokines, acting on the T cells that produce them in an autocrine fashion, as well as modulating responses of other cells through paracrine pathways. Although stimulated $CD8^+$ CTL also secrete lymphokines, the array generally is more restricted, compared to HTL.

$CD4^+$ T cells have been subdivided further into two distinct subsets based on the pattern of lymphokine secretion; T_H1 cells produce IL–2 and IFN-γ (and other lymphokines) but not IL–4, IL–5, IL–6, or IL–10, while T_H2 cells produce IL–4, IL–5, IL–6, and IL–10 (and other lymphokines) but not IL–2 or IFN-γ (1). The functional activities of these subsets also are different: T_H1 cells mediate delayed-type hypersensitivity while T_H2 cells provide efficient "help" for B lymphocytes (1). The dominance of particular $CD4^+$ subsets also appears to influence significantly the characteristics of some immune responses in vivo. For example, in experimental leishmania infections in mice, a T_H1-type response predominates in mouse strains that are resistant to infection while a T_H2-type response predominates in mouse strains that are susceptible to progressive infection (2)

Not all $CD4^+$ T cells are encompassed within the T_H1 and T_H2 designations. The pathways that lead to the development of T_H1 and T_H2 cells, which appear to represent polar extremes among T cell clones, have not been well characterized. $CD4^+$ T cell clones that secrete other arrays of lymphokines appear rather frequently (3–5). Naive $CD4^+$ T cells may secrete only IL–2 after initial stimulation, and cells which secrete IL–4 are

found infrequently, if at all, among naive $CD4^+$ T lymphocytes (6). The various $CD4^+$ subsets may arise from a common precursor (7, 8). Some of the factors that influence selectively the development of $CD4^+$ T lymphocyte subsets following initial stimulation with antigen have been defined (6). The following discussion will consider primarily those mechanisms that selectively affect T_H1 and T_H2 subsets. These processes are likely involved in the shaping of the characteristics of immune responses as they develop following immunization. Almost certainly they are involved in maintaining particular patterns of established immune responses.

It is difficult to define and characterize mechanisms involved in the activation of T lymphocytes using cells obtained directly from animals because lymphoid tissues consist of a heterogeneous mixture of cells. Several kinds of clonal populations of T cells have been used in order to simplify the model systems. Tumor cell lines and T cell hybridomas, produced by fusing a T cell tumor with a normal T lymphocyte, have been used with great success to study some events associated with T cell activation. It is easy to obtain large numbers of such cells. However, neither T cell lymphomas nor T cell hybridomas are completely satisfactory for studying the regulatory processes that control T cell functions since both types of cells, because of their neoplastic nature, are inherently regulated abnormally. Fortunately, regulatory mechanisms can be studied rather readily using clonal populations of T cells that retain "normal" phenotypic characteristics. It has been possible to derive and maintain murine and human T cell clones that react with specific antigens and that retain stable patterns of lymphokine production and function, apparently indefinitely. Such clones usually do not manifest evidence of malignant transformation. These clones provide useful model systems for evaluating regulatory mechanisms that may affect T lymphocyte subsets differentially.

We have defined several distinct mechanisms that affect differentially the activities of murine T lymphocyte clones representing various $CD4^+$ and $CD8^+$ subsets. This review emphasizes our studies, but we attempt to relate our observations to those of other investigators who have described other immunoregulatory events.

REGULATORY INFLUENCES THAT AFFECT MURINE T LYMPHOCYTE SUBSETS DIFFERENTIALLY

1. *Interferon-γ (IFN-γ) inhibits proliferation of but not lymphokine production by T_H2 cells.* IFN-γ, at relatively low concentrations (50–100 units/ml), inhibits proliferation of murine T_H2 clones stimulated with

antigen, mitogens, or anti-T cell receptor (TCR) monoclonal antibody (mAb); however, secretion of lymphokines including IL–4 in response to these stimuli is not affected (9). IFN-γ also inhibits proliferation of murine T_H2 clones exposed to IL–2 or IL–4 (9, 10). IFN-γ does not affect proliferation or lymphokine production by T_H1 or other $CD4^+$ or $CD8^+$ clones that secrete IFN-γ. Although not absolute, the inhibitory effect of IFN-γ on proliferation of murine T_H2 cells is significant and seems to be sufficient to limit the clonal expansion of such cells. While IFN-γ does not inhibit lymphokine secretion by stimulated T_H2 cells, IFN-γ does inhibit many of the agonist effects of those secreted lymphokines. For example, IFN-γ inhibits proliferation of murine bone marrow cells stimulated with IL–3, IL–4, or granulocyte-macrophage colony-stimulating factor (GM-CSF) (11). In addition, IFN-γ can inhibit IL–4–dependent B cell differentiation (12, 13).

The mechanisms by which IFN-γ inhibits proliferation are not clear. The inhibitory effects of IFN-γ on proliferation are observed only in those T cell clones that do not secrete IFN-γ. Many T_H2 clones derived in other laboratories are dependent on IL–1, in addition to IL–2 or IL–4, for proliferation (14, 15). Furthermore, some T_H2 clones may secrete IL–1 (15). However, the inhibitory effect of IFN-γ on proliferation of T_H2 clones does not correlate with their dependence on IL–1 for proliferation. T_H2 clones derived in our laboratory are not dependent on IL–1, and they do not proliferate when exposed to exogenous IL–4, either alone or in the presence of IL–1. Their proliferation following stimulation of the TCR is dependent on IL–4 as indicated by the inhibitory effect of anti-IL–4 mAb. Nonetheless, IFN-γ inhibits TCR-induced proliferation of our T_H2 clones. IFN-γ does not inhibit early expression of the proto-oncogenes c-*fos* and c-*myc* induced by IL–4 or the increased expression of the IL–2 receptor induced by IL–1 (T. Gajewski, unpublished observations). It also does not cause increased levels of $[Ca^{2+}]_i$ nor does it activate protein kinase C or elevate intracellular cAMP. IFN-γ exerts its effects on proliferation of T_H2 clones relatively late, still causing significant inhibition of proliferation if added 24 hr after addition of IL–4 (T. Gajewski, unpublished observations).

Thus, IFN-γ serves as an immunoregulatory molecule through which T_H1 or $CD8^+$ lymphocytes can interfere with both the clonal expansion and the effector functions of T_H2 cells.

2. *IL–10 inhibits antigen-presenting cell (APC)–induced lymphokine production by T_H1 cells but not by T_H2 cells.* IL–10, a lymphokine secreted by T_H2 cells, B cells, and LPS-stimulated macrophages, inhibits lymphokine production by T_H1 clones but not by T_H2 clones (16). This inhibition is

somewhat selective in that secretion of IFN-γ and IL–3, lymphokines secreted mainly at later times after stimulation, is substantially inhibited while secretion of GM-CSF and LT/TNF, lymphokines secreted mainly at early times, is inhibited slightly if at all; the effect on IL–2 production often is less than the effect on IFN-γ production (16). IL–10 also inhibits the synthesis of IFN-γ by $CD8^+$ CTL clones, although the extent of inhibition is somewhat lower than that observed with T_H1 clones (17). Because IL–10 inhibits secretion of IL–2 by T_H1 cells and IL–2 causes proliferation of $CD8^+$ cells through a paracrine pathway, T_H2 cells and other cells that secrete IL–10 regulate clonal expansion of those subsets that produce IFN-γ.

Although there appears to be reciprocal regulation of responses of T_H1 and T_H2 clones by lymphokines secreted uniquely by these subsets, the site of regulation differs. As noted above, IFN-γ, secreted by T_H1 cells or $CD8^+$ CTL, apparently can act directly to inhibit the ability of T_H2 cells to proliferate in response to IL–4 secreted following stimulation of the TCR or to exogenous IL–2 or IL–4. However, IL–10 apparently exerts its effect indirectly by acting on APC to reduce their ability to stimulate lymphokine production by T_H1 clones (18). IL–10 acts preferentially on macrophages and does not affect the ability of B cells to stimulate lymphokine secretion by T_H1 clones; however, IL–10 does not affect the ability of either macrophages or B cells to stimulate T_H2 clones (18). The mechanism by which IL–10 affects macrophages is not certain, although it does inhibit production of IL–1, IL–6, and TNF-α by activated macrophages (19). Also, IL–10 inhibits lymphokine production by T_H1 cells only if metabolically active macrophages are used for stimulation; although fixed cells are less effective in stimulating lymphokine production by T_H1 clones, IL–10 has no effect if fixed antigen-pulsed macrophages are used (18). B cells also can secrete IL–10 (20) as can peritoneal macrophages that have been stimulated with lipopolysaccharide (LPS) (19). However, IL–10 secretion by LPS-stimulated macrophages is inhibited by IFN-γ (19). Thus, it appears that the selective inhibitory effect of IL–10 on T_H1 and $CD8^+$ cells may be related to the type of APC that stimulates these T cell subsets as discussed below in Section 3.

3. *Murine T_H1 and T_H2 clones proliferate optimally in response to distinct antigen presenting cell (APC) populations.* A panel of T_H1 and T_H2 murine T cell clones, derived from the same preparation of lymph node cells after sensitization in vivo with ovalbumin (OVA), secrete lymphokines and proliferate well in the presence of OVA and whole spleen cells. However, purified splenic B cells stimulate optimal proliferation of T_H2 clones while adherent spleen cells stimulate optimal proliferation of T_H1

cells (21). Proliferation of T_H2 clones stimulated with OVA and spleen cells that were irradiated with 3300 rad is dramatically less than that observed in response to OVA and spleen cells irradiated with 1000 rad; T_H1 clones respond similarly to OVA and spleen cells exposed to either radiation dose. Hepatic nonparenchymal cells, probably Kupffer cells, stimulate proliferation of T_H1 but not T_H2 clones (22). Differential activation of T_H1 and T_H2 clones does not correlate with the restricting element of the major histocompatibility complex or to susceptibility to inhibition by anti-CD4 or anti-LFA–1 mAb (21). Differential activation of T_H1 and T_H2 clones cannot be explained by differences in antigen processing by macrophages and B cells, because similar results are obtained when OVA or an immunogenic peptide from OVA is used (21). Production of IL–3 occurs when T_H1 or T_H2 clones are stimulated with either splenic adherent cells or B cells, indicating that both types of APC are able to induce at least partial activation signals in each lymphocyte subset. The failure to proliferate cannot be explained by suppression of growth factor production since T_H1 clones stimulated with antigen and B cells secrete nearly as much IL–2 as when stimulated with antigen and splenic adherent cells. Similarly, T_H2 clones secrete nearly as much IL–4 when stimulated with antigen and splenic adherent cells as when stimulated with antigen and B cells (21). It is not possible to restore optimal proliferation of either subset by adding either IL–1 or IL–6 to cultures (21).

This selective effect of different APC populations has not been observed in all situations by all investigators. Some apparent discrepancies may relate to the particular T_H2 clones studied. As noted above, some but not all T_H2 clones require IL–1 to proliferate in response to IL–2 or IL–4. Macrophages may provide a source of IL–1 in a form bound to the cell membrane (23), but dendritic cells and B cells apparently produce little if any IL–1 (15). Both macrophages and resting B cells have been reported to stimulate proliferation of both T_H1 and T_H2 clones, although exogenous IL–1 was required for B cells to stimulate T_H2 cells (24, 25). However, in these studies, peritoneal macrophages rather than adherent spleen cells were used as APC. It is likely that cell surface molecules important for cellular interactions or cell signaling either in T_H1 or T_H2 clones are differentially expressed, depending on source and prior treatment of APC populations (26). The antigen-presenting function of B cells is sensitive to radiation in excess of 3000 rads; radiation at this level apparently has little effect on antigen uptake and processing but leads to the loss of APC-dependent co-stimulatory signals (27). The observation that antigen-presenting capacity of macrophages is radioresistant (28) provides additional evidence for the differential expression of co-stimulatory molecules on B cells and macrophages.

Since T_H1 and T_H2 clones respond differently in response to different kinds of APC, it seems likely that these subsets utilize different cell surface receptors and ligands. The fact that these subsets also differ in their susceptibility to the induction of anergy, as discussed below, may relate to requirements for different intercellular interactions or may reflect fundamentally different signaling events in T_H1 and T_H2 cells.

4. *T_H1 and T_H2 clones utilize different TCR-associated signaling pathways.* Stimulation of the TCR has been shown to result in hydrolysis of PIP_2 to yield diacylglycerol and IP_3 (29), an enzymatic reaction believed to be mediated by phospholipase C. Diacylglycerol presumably activates protein kinase C, while the IP_3 generated is thought to cause an elevation of $[Ca^{2+}]_i$ (29). However, these events appear to occur only in some but not all T cell subsets. Stimulation of T_H1 clones with concanavalin A (Con A) or anti-TCR mAb leads to elevated $[Ca^{2+}]_i$ and to the generation of inositol phosphates. However, these second messengers are not detected following stimulation of T_H2 clones (30, 31), even though T_H2 clones can be stimulated to secrete lymphokines by treatment with active phorbol esters and calcium ionophores, agents that mimic the effects of these second messengers (30). Also, treatment with ionomycin alone induces IL-4 production in T_H2 clones (32), while such treatment induces anergy in T_H1 clones (33). $CD8^+$ CTL apparently utilize signaling pathways similar to those of T_H1 clones (34). However, proliferation, but not lymphokine secretion, is induced in $CD8^+$ CTL by treatment with phorbol esters alone (35).

The inhibitory effect of high concentrations of anti-CD3 mAb on IL-2-dependent proliferation of T_H1 but not T_H2 clones, described below in section 5, may be related to these signaling differences. When T_H1 clones are stimulated with high concentrations of anti-CD3 mAb in calcium-free medium, dramatically less inhibition is observed. However, IL-2-dependent proliferation of both T_H1 and T_H2 clones is inhibited by calcium ionophore (36), suggesting a role for elevated $[Ca^{2+}]_i$ in this phenomenon.

The apparent utilization of different TCR-associated signal transduction pathways by T_H1 and T_H2 clones suggests that these subsets might respond differentially to various pharmacologic agents. Lymphokine production by T_H1 clones is substantially more sensitive to the inhibitory effects of cholera toxin (30, 37), cyclosporin A, and 8-Br-cAMP than is lymphokine production by T_H2 clones (30). Prostaglandin E_2, which elevates intracellular cAMP, has been reported to inhibit production of lymphokines by T_H1 clones but not by T_H2 clones (38).

5. *High concentrations of antigen (or anti-TCR mAb) inhibit IL-2-induced proliferation (but not lymphokine production) by T_H1 and CTL clones*

only. Most T_H1 clones exhibit a biphasic proliferative response when exposed to increasing concentrations of antigen (39–42). Proliferation of these cloned cells is markedly inhibited at high antigen concentrations. The failure to proliferate is due to an inability to respond to IL–2 since increasing levels of lymphokines, including IL–2, are secreted at high antigen concentration (42). It is striking that relatively low levels of lymphokines are secreted when T_H1 cells are stimulated with a concentration of antigen that causes optimal proliferation. Similar results have been obtained with a number of other T_H1 clones, but the concentration of antigen that is optimal for proliferation varies among clones, presumably reflecting differing affinities of TCR among the clones (43).

Since the affinity of the TCR appears to vary among clones, we utilized stimulation with immobilized anti-CD3 mAb to compare the effect of varying intensity of TCR stimulation among murine T cell subsets. Increasing concentrations of anti-CD3 mAb cause increased secretion of lymphokines by T_H1 and T_H2 clones, and the dose-response characteristics are quite similar (36). Similar results are obtained when T_H1 clones are compared with $CD8^+$ CTL clones (44). Although there are no apparent qualitative differences among the murine T cell subsets in their ability to produce lymphokines in response to stimulation with anti-CD3 mAb, there are striking differences in the proliferative response of the various subsets.

As is the case when stimulated with antigen, the dose-response curve for T_H1 clones is biphasic, and proliferation in response to high concentrations of anti-CD3 mAb, either alone or with added IL–2, is profoundly inhibited (36). Cells stimulated with high concentrations of anti-TCR mAb fail to proliferate in the presence of IL–2 even though they express increased numbers of high affinity IL–2 receptors (42). The failure to proliferate at high levels of anti-TCR mAb also does not reflect extensive death of cloned cells, since greater than 50% cell recovery was observed with most T_H1 clones even though they failed to incorporate ^{3}H-thymidine (T. Gajewski, unpublished observations). Comparable results have been reported by other investigators (45, 46). This inhibition is not due to soluble mediators, and anti-IFN-γ mAb does not reverse the effect. It should be noted that the proliferative response of T_H1 clones stimulated with immobilized anti-CD3 mAb in the absence of accessory cells is suboptimal; proliferation is augmented significantly when T-depleted syngeneic spleen cells are added to cultures (T. Gajewski, unpublished observations), an effect observed by other investigators (45, 46). However, the inhibitory effect of high concentrations of anti-CD3 mAb on IL–2–induced proliferation is only partially reversed by the presence of accessory cells.

With T_H2 clones, optimal proliferation is observed at about the same concentration of anti-CD3 mAb that yields optimal proliferation of T_H1

clones. However, at higher concentrations of anti-CD3 mAb, the proliferation of T_H2 clones remains at plateau levels. In striking contrast with the results obtained with T_H1 clones, high concentrations of anti-CD3 mAb either have no effect on IL–2-induced proliferation or actually cause augmented proliferation (36). The effect of immobilized anti-TCR mAb on IL–2–induced proliferation by $CD8^+$ CTL clones is generally similar to that of T_H1 clones (44). However, lymphokine-independent proliferation of $CD8^+$ cells induced by immobilized anti-TCR mAb (47) is not affected by high concentrations of such mAb; only the IL–2–induced component of the response is inhibited (44).

The striking differences in the effect of high concentrations of antigen or anti-CD3 mAb on IL–2–induced proliferation of T_H1 and T_H2 murine T cell clones provide additional evidence that at least some signaling pathways are different in these subsets.

6. *Exposure of T_H1 clones (but not T_H2 clones or $CD8^+$ CTL clones) to IL–2 induces unresponsiveness to antigen.* Pretreatment of T_H1 clones for 24 to 48 hr with concentrations of IL–2 similar to those produced by TCR stimulation renders these cells unresponsive to subsequent stimulation with antigen for a period of several days (48). Cells rendered unresponsive by IL–2 pretreatment express normal levels of TCR, CD4, and LFA–1, but they do not secrete lymphokines or proliferate (49). However, they remain able to proliferate to added IL–2 and to the combination of phorbol ester and calcium ionophore (49). This unresponsive state is characterized by failure to generate lymphokine mRNA as well as by decreased production of diacylglycerol and inositol phosphates in response to TCR ligation (34). The defect induced by IL–2-pretreatment appears to be proximal to the step in the post-receptor cascade that involves activation of phospholipase C (34). This type of unresponsiveness cannot be induced in T_H2 clones (36) or in conventional $CD8^+$ CTL (34).

IL–2–induced unresponsiveness represents a potent regulatory process which renders T cells that secrete IL–2 hyporesponsive for several days to further antigenic stimulation.

7. *T_H1 and T_H2 clones as well as $CD8^+$ clones can be cytolytic, but not all T cells use the same cytolytic mechanisms.* Although $CD8^+$ cells have often been designated "cytolytic cells" and $CD4^+$ cells have been designated "helper cells," we have found that many $CD4^+$ cells have cytolytic capabilities. Cytolytic activity was assessed using two different types of nucleated target cells. One was OVA-pulsed B lymphoma cells which functioned as effective APC; the other was Fc receptor-bearing cells to which anti-CD3 mAb had been bound for a retargeted assay. The majority

of T_H1 and T_H2 clones lyse both of these target cells with an efficiency similar to that of conventional $CD8^+$ clones (50).

Several cytolytic mechanisms have been suggested, including membrane injury induced by perforin molecules and a pathway that involves DNA degradation in target cells. Since sheep red blood cells (SRBC) do not have nuclei, lysis of SRBC apparently occurs by a mechanism that does not involve DNA degradation but presumably does involve membrane damage. The majority of T_H2 and $CD8^+$ T cell clones efficiently lyse SRBC coated with anti-CD3 mAb while T_H1 clones do not (50). This is the case even though anti-CD3-coated SRBC stimulate T_H1 clones to secrete IFN-γ (51). The ability to lyse SRBC efficiently correlates well with the ability to express perforin and CTLA–1 mRNA; mRNA for these molecules is easily induced in $CD8^+$ clones and in T_H2 clones that lyse SRBC but cannot be induced in T_H1 clones (50). However, some T_H1 and T_H2 clones are not cytolytic using any of the assay systems.

8. *$CD4^+$ clones from some mouse strains are not cytolytic if they do not secrete IFN-γ.* The failure of some T_H2 clones to exhibit cytolytic activity may be related to the mouse strain from which the clones were derived. Among clones derived from BALB/c mice, the vast majority that did not produce IFN-γ also did not lyse anti-CD3-coated target cells; these included clones that produce IL–2 only, IL–4 only, and IL–2 plus IL–4 (Table 1). In contrast, the vast majority of clones from BALB/c mice that did produce IFN-γ, were cytolytic; these included clones that produced IL–2, IL–4, or IL–2 plus IL–4 in addition to IFN-γ (M. McKisic, unpublished

Table 1 Proportion of OVA-reactive $CD4^+$ T cell clones from various mouse strains that express cytolytic activity

Mouse strain	T_H1	IFN-γ-producing	T_H2	Non-IFN-γ-producing
C57BL/6	3/4	27/28	10/12	30/33
C57BL/10	6/7	20/21	12/13	37/39
B10.A	2/3	11/13	10/19	15/16
B10.D2	1/1	23/24	18/25	48/63
DBA/2	2/4	10/12	2/5	7/12
BALB/c	16/18	47/50	0/14	0/33
BALB/c-H-2^{dm2}	2/3	31/33	0/5	2/22
BALB.B	2/4	10/15	0/3	1/30
BALB.K	2/3	31/37	0/2	1/36

Murine OVA-reactive $CD4^+$ T cell clones were harvested from microwell cultures and assayed for cytolytic activity using 2000 ^{51}Cr-labeled LK35.2 or LB27.4 tumor cells coated with anti-CD3-ε monoclonal antibody. Cells were incubated in V-bottom microtiter plates for 5 hr at 37°C, and the percent specific lysis was determined. Clones were considered to express cytolytic activity if the percent specific release was greater than 10%.

observations). Addition of IFN-γ to the cytolytic assay or to maintenance cultures did not induce cytolysis by non-IFN-γ-producing clones from BALB/c mice, nor did anti-IFN-γ mAb inhibit retargeted lysis by clones from BALB/c mice that produced IFN-γ, including T_H1 clones. The association between IFN-γ production and cytolytic activity was not related to the H–2^d MHC haplotype of BALB/c mice; clones derived from BALB.B and BALB.K mice as well as from BALB/c-H–2^{dm2} mutant mice have similar properties. Also, the vast majority of alloreactive clones derived from dm2 mice failed to lyse antigen-specific or anti-CD3-coated target cells if they did not produce IFN-γ. Among clones derived from C57BL/6, C57BL/10, B10.A, B10.D2, and DBA/2 mice, the majority of clones were lytic in retargeted assays, regardless of their lymphokine profile (Table 1 and M. McKisic, manuscript in preparation).

Thus, many T_H1 and T_H2 clones in addition to $CD8^+$ CTL have cytolytic capability, although they may not utilize the same cytolytic mechanisms. However, T_H2 clones and other $CD4^+$ clones from some mouse strains, notably those having the BALB/c background, are not cytolytic if they do not secrete IFN-γ. This characteristic needs to be considered in evaluating the functional effects of a predominantly T_H2 response in experimental infections in mice of this strain.

Implications of Differential Regulation of T Lymphocyte Subsets

Several parameters have been used to follow T cell activation. These include proliferation, lymphokine production, cytolytic activity, and generation of various "second" messenger molecules that are thought to be involved in signal transduction. Often, these various parameters have been assumed to reflect equally "T cell activation." We have documented above that several regulatory processes affect differentially these several parameters. In several situations, cell proliferation can be clearly dissociated from lymphokine production, and second messengers, usually coordinately expressed, can be modulated separately. Murine T_H1, T_H2, and $CD8^+$ T lymphocyte clones clearly respond differently to various regulatory influences. Some of these mechanisms serve to inhibit proliferation and thus limit clonal expansion of antigen-stimulated cells. Others serve to inhibit lymphokine production and thus regulate effector functions of other T lymphocyte subsets as well as other types of cells. The effects of the immunoregulatory processes discussed above on T_H1 and T_H2 clones are summarized diagrammatically in Figure 1.

Most T lymphocyte clones have been derived using cells obtained from relatively few anatomical locations. Almost all murine clones reactive with nominal antigens have been derived from the lymph nodes draining the

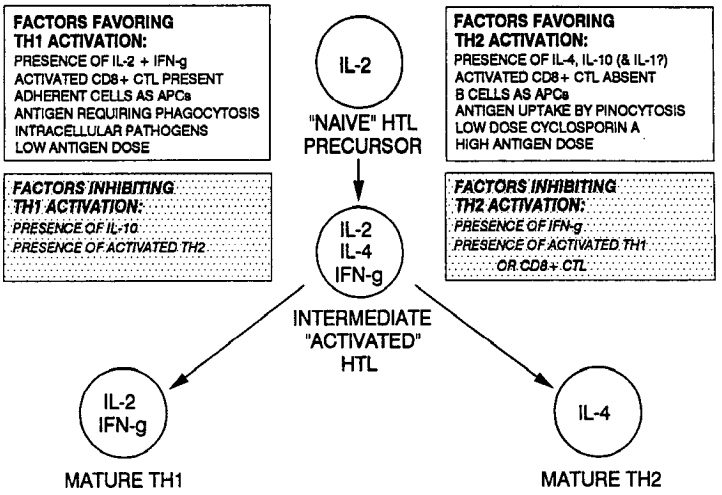

Figure 1 Factors predicted to favor or inhibit activation of T_H1 and T_H2 clones. [Modified from a figure published previously (36)]

site of primary sensitization. Murine alloreactive clones usually have been obtained from spleen cells. Most human T cell clones have been derived from peripheral blood lymphocytes. The APC studied have been from relatively few sources. Spleen cells have been most commonly used to stimulate murine T cell clones. The anatomical location of lymphocytes is determined in large part by the expression of cell surface molecules involved in homing to various lymphoid tissues (52). It is not yet clear if these homing receptors are differentially expressed on T cell subsets or whether functional characteristics of T lymphocytes are influenced by their anatomic location. Epstein-Barr virus–transformed B cell lines have often been used with human clones. The choice of APC often seems to have been made for convenience rather than for relevance to the situation in vivo. This is an important consideration since, as discussed above, T lymphocyte subsets respond differently to various types of APC.

It is not certain how results obtained with murine T cell clones relate to activation events initiated by stimulation of naive T cells with antigen, but at least some of the mechanisms that regulate the initial activation of $CD4^+$ T cells probably differ from those that control the reactivation of previously stimulated cells. Swain and colleagues have studied extensively the phenotypic and functional characteristics of $CD4^+$ T cells from normal mice and from mice that have been subjected to adult thymectomy; using these characteristics, they have defined properties of naive and memory T

cells. Their results, summarized in (6), indicate that naive T cells are contained within the short-lived, Mel–14^+, $CD45RB^+$, and Pgp–1^{lo} population of cells that disappears after adult thymectomy. After initial stimulation, naive T cells secrete only IL–2. These cells proliferate and develop by 4 days into effector cells that can be restimulated to secrete much larger quantities of several different lymphokines and to become Pgp–1^{hi}. Expression of Mel–14 is variable, and cells may lose expression of CD45RB (20).

Conditions following initial stimulation of T cells in intact animals apparently influence the nature of the effector cells generated and the kinds of lymphokines produced on restimulation (reviewed in 6). The presence of IL–2 leads to secretion of predominantly IL–2 and IFN-γ; very low levels of IL–4 are produced. IL–4 enhances the generation of effector cells secreting IL–4 and IL–5 and suppresses the development of IFN-γ-secreting effector cells. Exogenous IFN-γ enhances development of effector cells that secrete substantially more IFN-γ, somewhat more IL–2, and considerably less IL–4. This type of effector cell appears to be relatively short-lived. Memory T cells are contained within the Mel–14^-, $CD45RO^+$, Pgp–1^{hi} population of cells that persists after adult thymectomy. Somewhat surprisingly, these cells secrete only IL–2 after stimulation. They also develop into effector cells that can be restimulated to secrete large quantities of lymphokines, including IL–4. Conditions favoring development of memory T cells have not been defined. However, Swain and colleagues suggest that TGFβ may favor development of such cells, although Coffman et al conclude that IL–4 and IFN-γ are the only factors clearly involved in subset differentiation of $CD4^+$ murine T cells in vivo (53). Similar conclusions have been reached with human $CD4^+$ T cells (54).

Observations made with cloned cells generally are consistent with those obtained in studies of immune responses in intact animals. However, it is not clear whether T cell clones are descendants of naive or memory effector cells. A widespread but unstated assumption seems to have been that T cell clones probably are memory cells. However, the regulatory influences affecting T cell clones reviewed above also are consistent with such clones being effector cells which are descendants of naive T cells rather than of memory T cells. Although effector cells derived from naive T cells appear to be short-lived in vivo, their short life span may be the consequence of degradation and disappearance of most antigens encountered in nature. Repetitive stimulation with antigen in vitro may be able to cause continued proliferation and may enable such clones to be maintained in culture apparently indefinitely.

Some of the regulatory mechanisms discussed above may explain various

immunological phenomena. For example, the inhibitory effect of high concentrations of antigen on proliferation of T_H1 clones but not T_H2 clones may account for the observation that delayed hypersensitivity reactions are favored by low daily doses of antigen while high antibody responses are favored by high daily doses of antigen (55). Phenomena attributed to $CD4^+$ suppressor cells probably involve some of the immunoregulatory mechanisms described above. Effective antigen concentration appears to influence $CD4^+$ T cell subset development in ways that may not be immediately evident. Bottomly and colleagues have observed that mouse strains differ in the type of $CD4^+$ subset that develops following immunization with human type IV collagen (56). H–2^s mice mount a T cell proliferative response but do not generate antibodies, while H–2 congenic mice of several other MHC haplotypes, including H–2^b, mount a strong antibody response but develop no or only a very low T cell–proliferative response. An immunogenic peptide derived from the *a*2 chain of human type IV collagen is immunogenic in both H–2^s and H–2^b mice, and the immune responses generated in these two mouse strains are the same types as those developing following immunization with intact human type IV collagen. However, $CD4^+$ T cells from H–2^b mice are able to mount a proliferative response to the peptide if the mice are sensitized with a hundred-fold higher concentration of the peptide than is optimal for immunizing H–2^s mice. These investigators suggest that either the peptide is processed differently in the two strains or the affinity of binding of the peptide to the two different class-II MHC molecules differs. In any event, these results indicate that factors in addition to the amount of antigen administered appear to be able to influence the effective ligand density.

Immunoregulatory stimuli that do not act directly through the TCR also can profoundly affect the functions of T lymphocytes. Some of these stimuli are provided by cytokines. IFN-γ apparently acts directly on T_H2 cells to inhibit proliferation. IL–10 apparently acts indirectly by affecting macrophages to inhibit lymphokine production and consequent IL–2–mediated proliferation of T_H1 cells. Through these mechanisms, T_H1 and T_H2 cells cross-regulate each other.

Although an MHC/antigen complex usually is sufficient to stimulate T_H1 cells to secrete at least some lymphokines, additional cellular interactions are required for optimal proliferation. In the absence of additional signaling through co-stimulatory molecules, a state of unresponsiveness, termed "anergy," is induced (33). Anergy also has been induced in a $CD8^+$ CTL clone that also produced IL–2, (57). Anergy apparently can be induced only in cells that secrete IL–2; it cannot be induced in T_H2 cells or in $CD8^+$ cells that do not secrete IL–2, using treatments that are

effective in inducing this state in T_H1 clones. The basis for this difference in susceptibility of T cell subsets to anergy is not known. It is of interest that stimulation of T_H0 cells (which secrete IL–2, IL–4, and IFN-γ in addition to several other lymphokines) with anergic stimuli preferentially inhibits IL–2 production, thus leading to the development of several characteristics of T_H2 cells [T. Gajewski, unpublished observations and (58)]. The differences in signaling pathways in T_H1 and T_H2 clones noted above may relate to the differential susceptibility to anergy. An important feature of anergy appears to be the relatively selective impairment of IL–2 production; secretion of other lymphokines, including IFN-γ and IL–3, is not as severely inhibited (33). Biochemical pathways leading to production of various lymphokines have been incompletely characterized, but multiple nuclear-binding proteins are important for IL–2 secretion (59). The pathways for activation of different lymphokine genes do not appear identical because treatment with a calcium ionophore is sufficient to induce secretion of IL–3 by both T_H1 and T_H2 clones, while treatment with both PMA and a calcium ionophore is required for production of IL–2 by T_H1 clones (30, 60). A critical biochemical event in the induction of the anergic state appears to be an increase in $[Ca^{2+}]_i$ under nonmitogenic conditions (60, 61). However, failure to proliferate may be the essential requirement for the development of anergy. Indeed, anergy develops in T cells even when both TCR occupancy and co-stimulatory signals are provided if proliferation is prevented by the presence of anti-IL–2 and anti-IL–2 receptor mAb (61).

In addition to being an autocrine growth factor for T_H1 cells and some $CD8^+$ cells, IL–2 acts to impair temporarily the responsiveness of these cells to antigen, an effect not observed in T_H2 cells and in $CD8^+$ cells that do not secrete IL–2. Thus, although proliferation is induced by interaction of IL–2 with its receptor, which can be expressed by all subsets of T cells, this interaction has other consequences in cells that secrete IL–2. The basis for this difference is not known, but the communication between the TCR and the IL–2 receptor is clearly different in T cells that secrete IL–2 than in T cells that do not. Unresponsiveness induced by exposure to IL–2 differs from anergy in several respects even though it also can be induced only in T cells that secrete IL–2. Pretreatment with IL–2 inhibits production of all lymphokines (48), while in anergy, production of IL–2 is markedly decreased but secretion of IFN-γ and IL–3 is less affected, as noted above (33). IL–2–induced unresponsiveness apparently involves a different discordant impairment of TCR signaling because treatment with a calcium ionophore plus antigen restores responsiveness, while treatment with phorbol ester plus antigen does not (49). In contrast, increased $[Ca^{2+}]_i$

levels apparently are required for induction of anergy; a calcium ionophore alone is a potent inducer of anergy (33). Cells recover spontaneously from IL–2–induced unresponsiveness if left for several days in culture in the absence of IL–2 (48). Anergic cells do not regain responsiveness to antigen unless maintained for a prolonged period of time in the presence of IL–2 (33).

Immunoregulatory "co-stimulatory" signals appear to be provided by cell surface structures on antigen-presenting cells, and the relevant signals often can be provided by allogeneic non-T spleen cells (63). Co-stimulatory molecules include B7 (64) and heat-stable antigen (HSA) (65). The level of expression of these molecules by APC is likely to be influenced by the state of activation and the presence of various cytokines (33, 66). Interactions of these molecules with their receptors such as CD28 (64) and CTLA–4 (67), in conjunction with stimulation through the TCR, appears to be required for optimal activation of T_H1 cells. Other cell surface structures are involved in interactions between APC and T cells, and at least some of these molecules probably participate in signaling events in T cells. These structures include CD2, CD21, LFA–1, and probably other molecules as well (68–71). Although experimental results appear to vary among different laboratories, it is clear that not all APC are equally effective in stimulating all manifestations of T cell activation. Activated B cells behave differently as APC than do resting B cells (72), and peritoneal macrophages treated with IFN-γ function differently than do resident peritoneal macrophages (73). Some of these differences relate to differential expression of cell surface molecules involved in T cell adhesion and/or signaling. It is likely that the list of cell surface molecules involved in functional interactions between T cells and APC will continue to grow.

Signaling pathways in T lymphocytes have been incompletely characterized. It is evident that differences exist among the T cell subsets. Thus, although inositol phosphates and $[Ca^{2+}]_i$ increase after stimulation of the TCR of T_H1 and $CD8^+$ CTL, these events do not occur in T_H2 clones. Current interest is centered on the role of tyrosine phosphorylation in signaling in these various types of T cells. The functional distinctions of the various T cell subsets described above indicate that there probably are multiple differences in the signaling pathways utilized by these subsets. Understanding the mechanisms that differentially regulate the various kinds of T cells, in addition to providing insights into the molecular events associated with activation of those subsets, should facilitate modulation of their activities in vivo, making it possible to influence favorably the outcome of disease processes.

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IMMUNOLOGICAL MEMORY

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Abstract

The past five or six years has seen a resurgence of interest in immunological memory. Areas in which important advances have been made of late or in which problems in understanding persist are covered here: (i) Selection of virgin B cells for entry into the peripheral pool. (ii) Expression of immunoglobulin isotypes and other markers on memory B cells. (iii) Development of memory B cells as a separate lineage from primary response B cells. (iv) Sites of production of memory B cells. (v) Signals that rescue mutating B cells in germinal centers, forming the basis of affinity selection, and programming further differentiation. (vi) The myriad markers of memory T cells, in particular CD45R isoforms. (vii) Selective migration pathways of memory T cells and its possible molecular basis. (viii) The lifespan of memory cells and factors that influence their long-term survival. The data accumulated during this period which have vastly increased our understanding of memory have at the same time highlighted unresolved problems that could block further progress in the field. The thorny question that we cannot at present answer is: How does a memory cell differ from an activated cell and, in the case of T cells, from an effector cell? The problem bears on the interpretation of any study that sets out to correlate memory phenotype with memory function. Immunologists may have donned an intellectual straitjacket in their search for the memory cell.

INTRODUCTION

The immune system does not forget an antigenic insult. Its memory maintains the specialist tools it will require to deal efficiently with recurrent

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infection. Thus prepared, the immune system mounts an accelerated and enhanced response upon its second encounter with an antigen. We acknowledge memory as one of the defining features of the immune system in higher vertebrates.

At a cellular level we think that memory is the result of the generation of a population of antigen-specific memory cells that survive indefinitely in the immune system. Out of this notion arose the idea that memory cells should exhibit a characteristic phenotype (surface marker, biochemical, etc) that is different from virgin cells. In recent years, a host of putative "memory markers" have appeared on the scene. Some are more convincing than others, but we still do not possess the means to say whether a particular cell is a memory cell. A clear lesson from the search for "memory markers" seems to be that immunological memory is more complicated than we imagined; or is it? A view proposed by myself and others is that if memory were a function of continued stimulation rather than specialized cells then the "memory marker" would turn out to be a red-herring. This overstates the case, as there are clearly markers that have some functional correlation with cells that carry "memory;" however, a clearer understanding of the nature of memory will be gained only if we can know the intermitotic life span of memory cells and whether they require external stimuli to persist for long periods.

The field of immunological memory has seen a resurgence of interest in recent years. This is reflected in the number of reviews written; because of this, the reader will later be referred to areas covered in detail elsewhere. I hope in this review to provide an overview of the field as it stands at present and to highlight some of the exciting recent advances that increase our understanding of immunological memory.

WHEN DOES A NAIVE CELL BECOME A MEMORY CELL?

It is important to ask this question in the light of the fact that naive B cells, if they are to enter the stable, peripheral recirculating pool after emerging from the bone marrow, must undergo a process of selection that involves interaction of the combining site of their Ig receptors with molecules in their environment. This state of affairs is predicted purely by measuring the rate of bone marrow B cell production on the one hand and B cell lifespan in the peripheral pool on the other (the peripheral pool is taken to mean those B cells that inhabit follicles in lymph nodes, Peyer's patches, splenic white pulp, and other secondary lymphoid organs, i.e. mucosal associated tissues). Thus, 5×10^7 B cells exit the bone marrow every day (1, 2), enough to repopulate the peripheral pool in 4–5 days.

The peripheral pool, however, does not turn over within 4–5 days; the cells have an average lifespan of 4–6 weeks or longer (3–5). We can conclude that the majority of newly produced B cells die soon after exit from the marrow, and those that enter the stable, recirculating pool must have been selected in some way (5).

A more direct measure of selection is obtained by analyzing the V gene expression of the various pools of cells. It has been known for some time that the repertoire of V region expression in the bone marrow differs from that in the periphery (6, 7). This was always interpreted as the result of clonal expansion following antigen-driven responses in the periphery. More recently, Gu et al (8) have analyzed the V gene repertoire expressed by IgM^+ IgD^+ B cells, that make up more than 90% of the peripheral, recirculating pool and that are considered naive. While the expression of J558 genes by IgM^+ IgD^+ B cells in the bone marrow was random, IgM^+ IgD^+ cells in the spleen exhibited a clustered expression. Both of these populations (in bone marrow and spleen) are viewed as naive cells; however, in their passage from bone marrow into the splenic white pulp, the IgM^+ IgD^+ cells appear to have been "selected." This has been termed "ligand-selection," and based on preliminary results from germ-free (but not antigen-free) mice, the authors favor a role for external antigen (possibly from the gut) in the selection process.

Can we go on calling these cells naive or virgin cells? If they are not naive, are they memory cells? They do not possess two of the characteristics of true memory B cells: (i) clonal expansion, and (ii) somatic mutations. As we shall see, the fact that they do not express IgG does not necessarily rule them out as memory cells. For the time being, "ligand selected" seems to be an accurate if cumbersome appelation. Only later when these cells become involved in a T-dependent immune response do they undergo the changes characteristic of true memory cells.

Are T cells different in this respect? As no systematic analysis of the peripheral "naive" T cell V gene repertoire has been carried out, we cannot know the answer to this question. The only data that may pertain indicate that antigen has an influence on the representation of particular V regions in the periphery (9).

IMMUNOGLOBULIN ISOTYPES ON MEMORY B CELLS

Until recently very few surface membrane molecules other than immunoglobulin (Ig) showed any promise of differentiating naive from memory B cells. It is natural therefore that, due to the heterogeneity of expression on peripheral B cells, the surface Ig isotype was pursued as a marker of

memory cells. The literature on this subject is confusing. In particular, a flurry of papers in 1979 and 1980 tend to contradict each other on the importance of IgD^+ and IgD^- memory B cell populations (10–13). The experiments of Vitetta and coworkers (10) suggested that upon adoptive transfer only IgD^+ cells (and not IgM or IgG positive populations) could propagate memory and self-renew. In contrast, the Herzenbergs' group, using very similar FACS-sorting and adoptive transfer experiments, proposed that while IgD^+ cells could transfer memory, they did not self-renew but gave rise to IgD^- memory cells which were self-renewing and could continue to propagate memory (11, 12). In addition, they showed that the IgD^- cells gave rise to much higher affinity antibody responses than the IgD^+ cells. In an earlier paper, using different methodology (stripping of cells with isotype-specific antibodies as protection from radioactive antigen-induced suicide), Coffman & Cohn (14) concluded that the predominant isotype on memory cells was IgG. During this period reports also suggested that memory cells could still express IgM on their surface (15), that they might express multiple isotypes (13), and that IgG^+ memory cells could still secrete IgM (16). What are we to make of this? It is difficult to find obvious fault or differences in the FACS-transfer experiments despite uncorroborated statements that a particular antigen or immunization protocol will yield mostly IgD^+ memory cells (11). Some of these questions might usefully be reexamined.

Later experiments also address the question; Hayakawa and colleagues sorted antigen-binding cells (using the fluorescent dye phycoerythrin as the antigen) and put them into culture with antigen and T cells and looked for antibody production (17). They found that most of the IgG1 antigen-specific antibody came from IgM^- IgD^- cells. To my mind this is not necessarily a demonstration of memory but more an indication that this particular population is in a state to differentiate and secrete antibody when cultured with T cells, while the IgM^+ IgD^+ cells are not. It seems possible that what these workers identify is not a memory cell but a cell that has already started to differentiate into a plasma cell, deleting IgM and IgD and now expressing only IgG. Nevertheless, the current dogma is that memory resides with an IgG^+ population (or IgA or IgE in the case of responses of these classes) and that cells carrying IgM or IgD contribute little to the memory pool (17–19).

If this is true then a conundrum arises (20): The proportion of IgG1 memory B specific for a particular antigen is quoted as 0.01–0.07% of splenocytes (a figure derived by fluorescence staining of antigen specific cells; 17–19). If we assume that the techniques are sensitive and accurate and that these figures are not underestimates, this translates to 0.02–0.14% of splenic B cells. When we include memory cells expressing other IgG

subclasses (ie. IgG2a, IgG2b) the figure reaches 0.06–0.42% of splenic B cells that are specific for any one antigen. Even if it decays with time (see later) we know that memory can be very long-lasting; often for the life of a mouse. If we assume, even as a conservative estimate that an adult mouse will encounter 100 antigens during its lifetime, then this mouse should have 6–42% IgG^+ B cells in its spleen. The number of IgG^+ B cells in an adult mouse spleen is usually only 1–2% (19, 21, 22, and personal observation), a level that does not increase with age. If, on the other hand, IgM^+ IgD^+ cells were also memory cells the puzzle would not arise, and indeed we might have an explanation for why the peripheral B cell pool is filled with relatively long lived (4, 5, 8) IgM^+ IgD^+ cells that are thought at present to be naive. The scheme of differentiation for memory B cells that I am proposing is based on the idea that the immunoglobulin isotypes expressed on a memory B cell may fluctuate as a result of the variety of signals it may meet. The scheme shown diagrammatically in Figure 1 owes

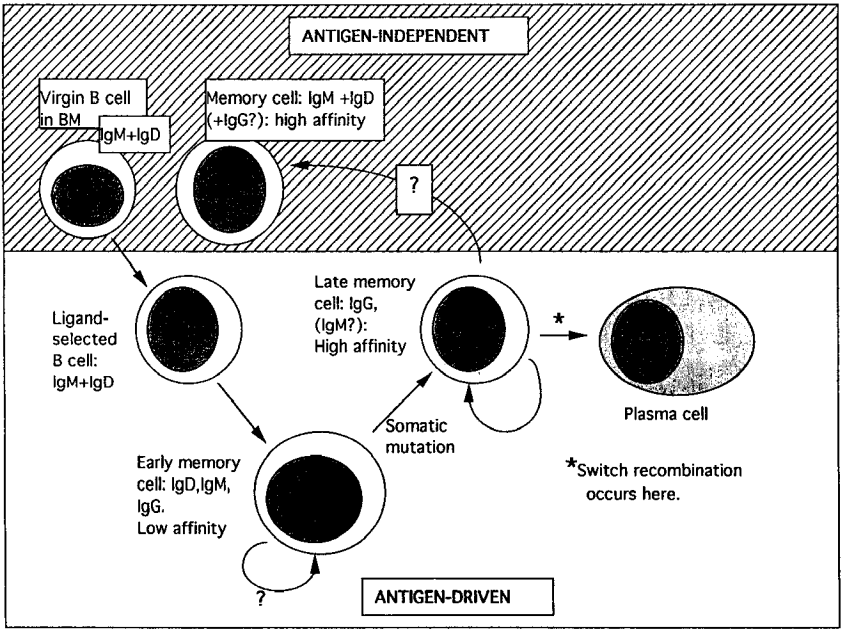

Figure 1 Scheme of B cell differentiation with particular reference to immunoglobulin isotype expression. A synthesis based largely on published data in which the proposed reversion of IgG-bearing memory cells to an IgM^+ IgD^+ phenotype is speculative; there is no direct evidence for this pathway. Circular arrows next to cells indicate a capacity to self-renew, not cell division. This diagram owes much in style and content to one contained in Ref. 11.

much to one drawn originally by Herzenberg and colleagues (11), but with some important speculative additions: In particular the heterodox proposal that an IgG^+ memory B cell can reexpress IgM and IgD.

The idea that memory cells might express multiple isotypes or revert from expression of IgG to IgM and IgD relies on the demonstration that expression of upstream isotypes can be brought about by mechanisms that require no deletion of the DNA between the V gene and the heavy chain C region in use. The most likely and well-documented mechanism is the production of long mRNA transcripts containing all of the requisite C region genes (23–26). The direct demonstration of long transcripts in normal splenic B cells (24) does not seem to be open to the criticism of other studies (23) that the double expression is due to cytophilic absorption of Ig (27, 28). If all of the C region genes are maintained until a very late stage in memory cell differentiation, I would propose that in the absence of further stimulation a memory B cell may revert to its naive phenotype.

OTHER MARKERS OF MEMORY B CELLS

Probably the first nonimmunoglobulin marker that was proposed to distinguish naive and memory B cells has turned out to be the most convincing and reliable. This is the heat stable antigen as recognized by the monoclonal antibody J11D (29). It is likely that another antibody reported as making this distinction (NIMR–2; 30) also had the same reactivity (M. Parkhouse, personal communication). The subsequent work on populations of B cells separated on the basis of J11D reactivity (31–33) has shown that, after adoptive transfer into SCID mice as well as in splenic fragment assays, $J11D^{hi}$ cells take part only in primary antibody responses and $J11D^{lo}$ cells only in secondary antibody responses. In other words the precursors of secondary response memory B cells, express almost no J11D, while primary B cells have high levels. The functional identity of J11D lo and hi populations has been underscored by the finding that somatic mutations are found only in the Ig V regions of $J11D^{lo}$ cells (31) and that these cells, in contrast to $J11D^{hi}$ cells, give rise to germinal centers upon transfer (33). These are both characteristics of memory B cells.

Few other non-Ig molecules are reported as stable markers of memory B cells. CD44, a lectin-like adhesion molecule, is expressed at higher levels (34). Other adhesion molecules such as LFA–3, ICAM–1, and B7 are involved in B-T cell interactions and exhibit co-stimulatory effects for the T cell and B cell (35–40). In addition, the B cell adhesion molecule CD22 is known to interact with CD45R0 on memory T cells (41). As yet these molecules have not been shown at elevated levels on memory B cells, although I suspect this is because no one has looked specifically at memory

cells. Some molecules are known to be up-regulated following B cell activation; B7, Blast1, CD5, CD23 (see 42 for review). Two other markers distinguish virgin and memory cells, but only at a very specific point in their development, namely in germinal centers: These are MEL 14 (L-selectin) and peanut agglutinin (PNA). B cells as they begin to proliferate in germinal centers become PNA^+ and $MEL\text{–}14^-$; after this phase the selected population that rejoins the recirculating pool as memory cells becomes PNA^-, $MEL\text{–}14^+$ again (43, 44). Thus, cells that transfer memory responses 7–14 days after immunization will be PNA^+, $MEL\text{–}14^-$ while several weeks later they will reside in the PNA^-, $MEL\text{–}14^+$ population (43, 44).

GENERATION OF MEMORY B CELLS

Is There a Memory Cell Lineage?

There are three possible models to explain the development of memory cells: (i) They are derived from an "unequal" division of a precursor cell, giving rise to a memory cell and a plasma cell. (ii) The clonal progeny of the precursor cell come under the influence of different lymphokines, T cells, or accessory cells and hence differentiate into either memory cells or plasma cells. (iii) Memory cells arise from a completely different lineage from the cells that take part in primary immune responses. Reports of the restricted use of V genes (idiotypes) in primary responses that disappear in the secondary have been seen as support for the lineage hypothesis; for instance, the T15 idiotype dominant in the primary antiphosphorylcholine response is absent in the secondary (45), and a similar phenomenon is apparent in the response to 2-phenyloxazolone (46). In the primary response to NP ([4-hydroxy–3-nitro-phenyl] acetyl) antibodies bear λ light chain, while in the secondary there is an almost absolute switch to κ-bearing antibodies (46). These observations might just as easily be interpreted as the result of clonal selection of higher affinity antibodies. The lineage hypothesis has been viewed until recently with some scepticism. However, as we indicated in the previous section, the marker J11D provided the facility to isolate from naive, unimmunized mice precursor cell populations that had quite different characteristics in primary and secondary adoptive responses (31). To reiterate, $J11D^{hi}$ cells make almost all of the primary antibody response and do not take part in the secondary, while $J11D^{lo}$ cells only make antibodies in the secondary response. The recent observation that the somatic mutation process starts very early after immunization (E. Källberg, T. Leanderson, and D. Gray, manuscript in preparation; see later) lends weight to the idea, with its implication that immediately after antigenic encounter some cells start to proliferate and

mutate to give rise to the memory pool. While it can be argued that this state of affairs could arise stochastically from a single precursor population, which under the influence of a certain sets of signals becomes either memory cells or plasma cells, it is difficult to resist the lineage argument if cells can be separated into memory precursors prior to immunization (31–33).

Sites of Production of Memory B Cells

The role of the germinal center in the generation of memory cells has been established for some time (48–50). Klaus & Humphrey (49) prevented the induction of the germinal center reaction by treatment of mice with cobra venom factor and in doing so inhibited the development of a secondary response. Coico et al (44) purified germinal center B cells based upon their reactivity with PNA and looked at their capacity to transfer memory responses to adoptive hosts; they found that at early times after immunization (i.e. 7–14 days, when germinal centers were present in the lymphoid tissues) memory could be transferred only with the PNA^+ population. Thereafter, it was carried with the PNA^- cells, illustrating the fact that memory cells generated in the proliferating, nonrecirculating germinal center population reenter the recirculating pool of B cells. However, as we point out later, the number of cells produced in the germinal center vastly exceeds the number leaving, the result of stringent selection processes.

The improvement of immunohistological techniques, over the past few years, has led to an increased understanding of the dynamics of memory B cell development. It is now possible to stain simultaneously on the same section for antigen-binding cells, cell surface markers, and incorporation of bromodeoxyuridine (BrdU) to assess cell turnover (51, 52). This sort of analysis, following injection of thymus dependent antigens, has revealed at least two distinct phases of B cell activation. The first occurs very early, peaks towards the end of the first week, and occurs in T cell zones such as the periarteriolar lymphocytic sheath (PALS) of the spleen (51–53). The second phase, which should be seen as a natural progression of the late primary response, occurs as B cells start to proliferate within follicles. This proliferation is antigen-driven and may be dependent upon the deposition of antigen as immune complex on the follicular dendritic cells (FDC) (48–50, 54) that form a structural network within the germinal center and primary follicle (55–57). It is clear, however, that the cell that enters and proliferates has already been activated at an extrafollicular site (58, 59).

In the spleen the first proliferating foci within follicles appear at around 7–8 days after immunization (51–53), while in lymph nodes this may be accelerated by 3–4 days (60; E. Källberg, T. Leanderson, D. Gray, manuscript in preparation). Over the next week the germinal center reac-

tion progresses through a series of quite distinguishable stages that have been defined by MacLennan and colleagues (52, 61–63). In brief, the early homogeneous proliferation of B blasts (no CD39, no IgD, but low levels of other isotypes) gives rise to centroblasts (CD38, CD10 positive, no sIg at all). Centroblasts have a very rapid cell cycle time (6–7 hours; 64, 65) and soon give rise to smaller cells, which come out of cell cycle and start to reexpress low levels of Ig, usually IgG isotypes or IgA (66, 67). It is these centrocytes that appear to be the immediate precursors of the memory cells that rejoin the recirculating pool. As we discuss later, centrocytes go through several selection processes before this can happen.

The cells that do make the transition and leave the germinal center as memory cells seem to make quite distinct choices as to the anatomical site that they will inhabit. A large proportion of memory cells enter the recirculating pool, distributing themselves throughout the lymphoid system; although a certain "homing" potential can be observed, for instance as a result of retention of IgA-bearing cells in gut-associated tissue (68). The other major site where memory B cells are found is in the marginal zone of the spleen (52, 69). Following antigen-specific proliferation in germinal centers, antigen binding cells start to colonize the marginal zone. The marginal zone memory cells like all marginal zone B cells are sessile, i.e. not part of the recirculating pool, although their relationship (precursor-product) with recirculating memory B cells is unclear (69, 70). Upon secondary antigenic stimulation both populations begin to proliferate: (i) Recirculating memory B cells proliferate in follicles, although they do not form germinal centers. (ii) Marginal zone memory B cells migrate into the PALS where they proliferate. The specialized role of these two populations remains obscure.

AFFINITY MATURATION

Somatic Mutation

At the start of this chapter I talked about "enhanced" memory responses; in real terms this means that the memory B cell response is of higher affinity than the primary response. At least two mechanisms are responsible for the increase of antibody affinity during the course of a response. The first is a selection of high affinity V_H/V_L combinations from the vast primary repertoire expressed by naive B cells and reiterated frequently because of the extremely high rate of production from the bone marrow. In other words as antigen levels fall, only cells with high affinity receptors will continue to expand and so to dominate the response. Superimposed upon this in mammals is the process of somatic hypermutation of V genes. This is due to an obscure molecular mechanism that introduces point

mutations into V regions. Whether as the result of selection or due to targetting of the responsible enzyme, the majority of mutations appear in the complementarity determining regions (CDR) that form antigen-binding areas of the V region. Data detailing the pattern of nucleotide replacements, the timing, the affinity increase, what can be implied about selection and the clonal history of mutations forms a very large literature in this field (71–79). I do not deal with these aspects in any great detail here but refer you to several good recent reviews (80, 81, and the whole of volumes 96 and 105 of *Immunological Reviews*).

It is probably fair to say that most if not all B cells that become part of the memory pool have undergone somatic mutation (19, 81). As such, mutated V genes are at present the only reliable marker of memory B cells. I would like to consider here how the mutation process fits into the scheme of memory cell development as we understand it. Some years ago it was noted that the appearance of the first somatic mutants in the spleen correlated with the development of germinal centers in that tissue (82). The high rate of cell division (6–7 hours; 65), giving ample opportunity for the introduction of point mutation during DNA replication and a potential means to select efficiently mutated cells (see later), made this proposal entirely plausible, and it was quickly accepted in the literature (77, 83, 84). It is only recently, however, that direct proof of ongoing somatic mutation in germinal centers has been forthcoming (85, 86; E. Källberg, T. Leanderson, D. Gray, manuscript in preparation). This has been most convincingly demonstrated by the ingenious method of scraping germinal center cells from frozen tissue sections (a much more reliable way of identifying them than PNA sorting), preparing DNA, and amplifying specific V region segments used in the response to NP (86). What is not clear from these studies is whether germinal centers represent a specialized site or a specific stage of B cell differentiation in which somatic mutation occurs. The detection of somatic mutants as early as day 4 (E. Källberg, T. Leanderson, D. Gray, manuscript in preparation), day 5 (87) or day 6 (88), around the earliest time at which germinal centers can be identified as histologically discrete entities, suggests that the mutation mechanism may be switched on even earlier. However, small foci of proliferation can be noted in follicles very early after immunization (approximately 2 days in carrier primed animals; I. C. M. MacLennan, personal communication) that may represent the expansion of germinal center precursor cells, cells of the memory lineage that are already mutating.

We know little of the signals that initiate germinal center proliferation or somatic mutation although the dissection of the germinal center reaction in vitro is underway (89, 90). Both phenomena are dependent upon T cell–

derived signals; neither occur in nude/athymic animals (91, 92) or as a result of stimulation with thymus independent antigens (93).

It is also not known whether memory cells undergo more than one round of somatic mutation. Evidence is lined up for and against the notion, none of it entirely convincing. Memory cells of defined mutation status have been adoptively transferred, restimulated, and reanalyzed (77). No new mutations were detected, but some doubts remain because the restimulation of the transferred cells involved not hapten-carrier (i.e. antigenic stimulation) but anti-idiotypic stimulation (77). The analysis of tertiary and quaternary responses shows an increased number of mutations with each additional immunization. However, it is unclear in these studies whether the mutations detected at 3 days after the boost in the tertiary response were already present before this boost, and possibly even 1 week after the secondary boost, because no samplings at intervening timepoints were made (84, 94, 95). An observation that may have some bearing on this controversy is that memory B cells do not seem, to any large extent, to participate in germinal center reactions. This conclusion arises out of the following experiment to assess primary and secondary antigen-specific B cell proliferation: Thus 6 weeks after immunization with KLH (keyhole limpet hemocyanin), when KLH-specific memory cells can be detected in the marginal zones and follicles, rats are boosted with DNP-KLH (DNP = 2, 4 dinitrophenyl). KLH-specific proliferation occurs in T zones and follicles, but no KLH-specific germinal centers develop. In contrast, DNP-specific B cell proliferation occurring in the same sites leads to germinal center formation (51, 61). The conclusion is that virgin DNP-specific B cells give rise to germinal centers while KLH-specific memory B cells do not.

Affinity Selection

The rapid proliferation of the centroblasts within germinal centers gives rise to a vast excess of progeny, most of which, even based on numerical considerations, cannot survive (5, 82). Estimates based on cell labelling studies within the recirculating pool indicate that no more than 5–10% reenter this pool (5). The current view is that the centroblasts are proliferating and mutating and giving rise to a population of centrocytes that come out of cell cycle, reexpress surface Ig, and undergo selection. It seems likely that the initial selection of these cells is based upon their continued ability to bind antigen that is localized on the surface of FDC as an antibody-antigen complex (52, 61, 63). This hypothesis provides a rationale for efficient affinity selection; in general, only cells that have higher affinity for antigen than the antibody within the FDC immune complex will be stimulated (82).

Evidence in vivo and in vitro suggests that centrocytes are a labile population that die rapidly after coming out of cell cycle unless given a "positive" signal. In histological sections nuclear fragmentation is evident, as is the ingestion of pyknotic nuclear bodies by tingible body macrophages (57, 64). In vitro, most centrocytes isolated from tonsils undergo apoptosis within 24 hr of culture unless rescued (96). One efficient rescue signal is cross-linking of surface IgG by antibodies coated onto sheep red blood cells (96). This signal presumably mimics the signal delivered by antigen on the FDC via the centrocyte surface immunoglobulin. Thus antigen-binding cells are rescued for survival in the memory pool or for further differentiation to plasma cells. The signals that direct the cell along these pathways have also recently been elucidated by the analysis of the rescue of centrocytes in culture: Signals delivered via CD40 rescue centrocytes and leave them as small resting lymphocytes that appear phenotypically like memory B cells (61, 97, 98). In contrast, stimulation of the centrocytes with either IL–2 or soluble CD23+IL–1α results in differentiation to lymphoplasmacytoid cells (61, 63, 97, 98). The source of these signals may be from T cells or from accessory cells: The CD40 ligand is now known to be found in activated T cells (99) and in particular on CD4 T cells (P. Lane, A. Traunecker, S. Hubele, S. Inui, A. Lanzavechia, D. Gray, *Eur. J. Immun.* In press) with a phenotype typical of T cells found in and around germinal centers in B cell follicles (100, 101). These T cells are also the likely source of IL–2, while the CD23 signal seems to be delivered by subpopulations of FDC in the apical light zone that express it at high levels (see Figure 2).

With this knowledge and the increasingly sophisticated structural analysis of marker expression in germinal centers (61, 62), it is now very clear that the differentiation of cells through the germinal center until they emerge as memory cells is very ordered both chronologically and compartmentally. This is illustrated in Figure 2. It is important that mutated cells are selected first via their antigen-binding receptors. If other receptors, such as CD40, are engaged (as happens in vitro) all cells would be rescued irrespective of the antigen-binding affinity. The success of affinity selection relies upon the ordered progression of cells through distinct micro-enviroments, in a way similar to T cell development in the thymus. Just as in the thymus, a failure of this selection will have important consequences for self-tolerance, and indeed memory B cells are postulated to undergo a second phase of tolerance susceptibility (102).

All of the signalling pathways mentioned in relation to cell rescue seem to have their action through a biochemical pathway involving the proto-oncogene *bcl*–2 (97). The bcl–2 protein is normally expressed at high levels in most B cells, but it is lost in germinal center B cells (97, 103). Following

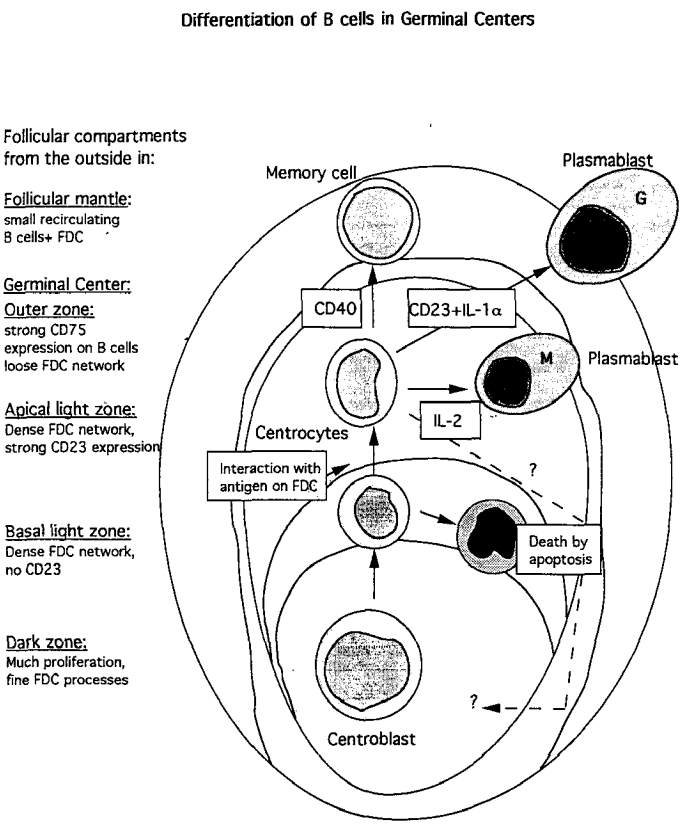

Figure 2 The compartments of secondary follicles in relation to B cell differentiation. Redrawn from two diagrams contained in Ref. 61. Note: Secondary follicles contain germinal centers; the follicular mantle of a secondary follicle is the same in its cell content as a primary, resting follicle.

in vitro rescue by the signals described (cross-linking anti-Ig, anti-CD40, or soluble CD23+IL–1α), germinal center cells upregulate levels of bcl–2 protein. It will be of interest to see if transgenic mice that constitutively overexpress the bcl–2 molecule (104, 105) carry out efficient affinity selection.

ACTIVATION REQUIREMENTS OF VIRGIN VERSUS MEMORY CELLS

An explanation often given for the accelerated and enhanced secondary response is that memory cells have lower activation thresholds/require-

ments than naive cells. Despite this belief the literature in this area is rather sparse. While both virgin and memory B cell responses require cognate, MHC-restricted T cell help, the amount required by memory cells seems to be lower; they proliferate in response to lower amounts of antigen, fewer T cells, or less lymphokine (106–108). This may be a reflection of the increased affinity of memory B cells for antigen or differential expression of lymphokine/T cell ligand receptors. Certainly, secondary B cell responses seem to be much more easily stimulated by noncognate bystander mechanisms (109, 110). So far as T cells are concerned, data are equally sparse in this area; however, there are reports of decreased activation thresholds for memory T cells (111, 112)

MEMORY T CELLS

Despite the recent accumulation of T cell markers that are used to enrich for cells that support "recall" or memory responses, the memory T cell has proved difficult to get a reliable handle on. The reasons for its elusiveness are probably two-fold: Firstly, T cells do not isotype switch, nor somatically mutate, nor proliferate in a defined anatomical site, and so we are left trying to define them in terms of the molecules that they put on their surface. Herein lies the second problem: Because many of these molecules turn out to be variably regulated, we cannot be sure whether the cells we are measuring are activated T cells, effector T cells, or memory T cells. Indeed we do not know if these are various manifestations of the same cell at different points in an antigen-driven cycle of stimulation. It may never be possible to isolate in a test tube cells of a single, well-defined phenotype that carries memory.

Descriptions of markers that are expressed at higher levels on memory T cells than on naive T cells have appeared twice in recent volumes of the *Annual Review of Immunology* (113, 114), and so although I provide a fairly exhaustive list of these molecules, I discuss in detail only some of them. Memory T cells express high levels of CD2, LFA1, LFA3 (115, 116), CD44 (113, 115, 117), Ta1 (118, 119), CD29 (120), VLA4, VLA5, VLA6 (121–123), CLA (cutaneous lymphocyte-associated antigen; 124, 125) and have lower levels of L-selectin (MEL–14; 126, 127) CD31 (PECAM; 123) and CD27 (128). In addition, alternative splicing of exons of the CD45 molecule leads to expression of various isoforms in T cells; the low molecular weight isoform (180KD) recognized only in humans by a monoclonal antibody (UCHL–1; 129, 130) was shown to characterize memory T cells (131, 132), and termed CD45RO. In other species, memory T cells have been isolated by their lack of staining for higher molecular weight forms of CD45 (133, 134).

The reports of differential lymphokine production by naive and memory T cells are contradictory and therefore confusing; this is entirely predictable unless everyone uses a large (and preferably the same) panel of markers to identify the particular subset or activation stage under investigation. The general concensus could be stated as follows; memory T cells (expressing $CD45R^{lo}$ = low molecular weight isoform) produce multiple lymphokines (IL–2, IL–3, IL–4, IL–6 and γIFN as well as TNFα and GM-CSF) rather like TH_0 cells, while naive cells ($CD45R^{hi}$ = high molecular weight isoform) produced mainly IL–2 (115, 135–139). The major dissenter from this view is the lab of Swain et al (140, 141). The correlation of expression of various isoforms with TH_1 and TH_2 cell types is at present obscure (142).

EXPRESSION OF CD45 ISOFORMS ON MEMORY T CELLS

At around the same time, studies in humans (131, 132) and rats (133) led to the definition of markers that would distinguish cells that, in vivo or in vitro, behaved like memory T cells. Thus, $CD45R^{hi}$ cells (recognized in humans by antibodies to CD45RA, in rats by OX–22 [that is, anti-CD45RC] and later in mice by antibodies such as 16A [that is, anti-CD45RB; 134] gave poor responses to recall antigens and were ineffective at providing help for B cells and antibody production. In sharp contrast, $CD45R^{lo}$ T cells (recognized in humans by the anti-CD45RO antibody, UCHL–1, and in rats and mice by lack of staining with OX–22 or 16A) provided help for B cells and antibody production and were highly active in assays of memory responses. While the presence on the cell surface of the $CD45R^{lo}$ isoform seemed to indicate memory function, it was also shown that human T cells expressed CD45RO very rapidly upon activation (132, 143, 144). CD45RA expression on activated T cells was lost much more slowly, and so cells expressed the two isoforms for some time (132, 143, 144). These data suggested that CD45RA T cells were the naive precursors of CD45RO T cells. This view was strengthened by two further observations. (i) In human cord blood there are only CD45RA T cells; CD45RO T cells only reach adult levels after 20 years (145). (ii) Adoptive transfer of rat $CD45R^{hi}$ T cells gave rise to $CD45R^{lo}$ T cells, whereas the $CD45R^{lo}$ phenotype seemed stable (146).

This apparent unidirectional pathway was worrying to some who maintained that expression of the low molecular weight form of CD45 by most thymocytes, which subsequently express the $CD45R^{hi}$ isoform upon export to the periphery (147–149), indicated there was every reason to suppose that the isoform expression was also reversible in mature, peripheral T

cells (150). This turns out to be the case, as Bell and co-workers demonstrated (151, 152): OX–22$^-$ (CD45R^{lo}) rat T cells can give rise, following adoptive transfer, to progeny that are OX–22$^+$ (CD45R^{hi}). Controls, including spiking the donor cell inoculum with OX–22$^+$ cells, rule out the possibility that the OX–22$^+$ cells arose by outgrowth of a small contaminating population of OX–22$^+$ cells. The experiments went one step further in showing that the phenotypic change was accompanied by a functional change in that T cells acquired graft-vs-host responsiveness, a characteristic of OX–22$^+$ and not OX–22$^-$ cells. Since this demonstration, human T cell lines have been reported to exhibit instability in CD45RO expression under certain culture conditions (153, 154).

While CD45R^{lo} may not be the lineage marker we initially believed, it clearly has value in defining functional "memory" populations. The significance of the reversion of memory T cells to a CD45R^{hi} phenotypic in functional terms is not easy to see. It may be a means of downregulating the immune response, in particular the antibody response (as CD45R^{lo} cells provide very effective help to B cells; it is of interest that the ligand for the B cell antigen CD22 is known to be CD45RO; 41). The functional aspects of this interconversion require more investigation; especially the question of whether memory cells that have reverted to a CD45^{hi} phenotype still function as memory cells—this seems unlikely in view of the inability to detect memory/recall responses in the CD45^{hi} population (131, 132).

MIGRATION PATHWAYS OF MEMORY T CELLS

The idea that memory cells might exhibit different migratory behavior to virgin cells is not a new one (155). Recent experiments in sheep have extended these ideas and indicate that there may be a multiplicity of different memory T cell subsets, based upon expression of adhesion molecules that allow extravasation into specific tissues (e.g. gut, skin) (156, 157). The initial observation was that T cells entering the popliteal lymph node in the afferent lymph (draining the hind leg) were almost wholly memory phenotype (CD45^{lo}) while those in the efferent lymph, which derive mainly from the blood, entering the lymph node across high endothelial venules (HEV) were mostly naive (CD45^{hi}) (156). This turned out to be true not only for lymphatics draining the skin but also for those draining gut tissues (157). Indeed there are many reports of tissue-specific homing of T cells; to the skin (124, 158), the gut (159, 160), inflamed synovium (161), and the lung (162).

How do these recent reports of tissue-specific homing of T cells differ from the much older reports of tissue-specific migration of activated cells or blasts (160, 163, 164)? The major difference is that the modern studies

insist that the cells under scrutiny are "memory cells" and not purely "activated cells." Advocates of the "selective homing hypothesis" propose that memory T cells with their increased levels of adhesion/accessory molecules do not require the specialized microenvironment of organized secondary lymphoid tissues for activation and can instead monitor tissues for reinvading organisms (121, 122). They are called memory cells because they express markers that have been associated with cells that give secondary responses in vitro or in vivo. But they are also activated cells, as all of the markers that define them are rapidly up-regulated upon activation; indeed $CD45R^{lo}$ cells are larger than $CD45^{hi}$ cells (165). In contrast, there is evidence that the small, resting T lymphocyte pool (naive and memory cells) exhibits random recirculation through HEV (166, 167). These authors (121, 122, 156, 157) do not deny the likelihood of a large number of memory cells entering lymphoid tissues via the "normal" route across HEV (122), but as yet have not assessed the relative size of the two memory pools nor any phenotypic and functional differences.

Others have claimed that this scenario fits better with the behavior of activated or effector T cells than with memory T cells (150). Thus, the role of activated/effector T cells is to seek out infecting organisms in inflamed sites and to deal with them, while the job of a memory T cell is to survey for reinvasion of these organisms after the inflammation/infection has subsided. It is pointed out that to cover the whole body surface area in search of rare antigens, at a time after the infection has subsided, is a very inefficient means of surveillance, especially when a very efficient system of lymphatics has evolved to bring antigens into lymph nodes and into the path of lymphocytes (naive and memory) that recirculate through from the blood. Whether these (121, 122, 156, 157) are studies of memory cells or activated/effector cells remains to be seen. A resolution might be at hand if we knew how to differentiate memory T cells from effector T cells.

THE MOLECULAR BASIS OF MEMORY T CELL HOMING

Whether the cells that home in a tissue-specific manner are true memory cells or activated/effector cells, it is interesting to consider the mechanism of their tropism. The older evidence suggests that T cells (or their progeny) that are activated by antigen in a particular tissue will return there because of the surface molecule expression induced in that site (168). Whether this is true is still not known. However, a lot is now known about the molecules that allow lymphocyte binding to endothelia in various tissues. There are several comprehensive reviews of this field (169–171). In this section I highlight some of these molecules that show differential regulation in

memory/activated T cells. Naive T cells and presumably memory T cells that cross HEV in lymph nodes use the interaction of L-selectin (LECAM–1, LAM–1 or MEL–14; 126, 127) with the vascular addressin recognized by MECA–79 (172). Of late the homotypic binding of CD31 (PECAM) has also been implicated in HEV-T cell interaction (123). On T cells that extravasate in tissues and enter lymph nodes via afferent lymphatics, the following molecules have been implicated: in skin: siayl LewisX or CLA (124, 125); in gut: HML–1 (173), LPAM–2 (174); in inflammed tissues: all of those already listed plus VLA–4, 5, 6 and LPAM–1 (122, 123, 174). The better known adhesion molecules such as CD2, LFA–1, LFA–3, and ICAM–1 have roles to play in allowing memory T cells to sample the endothelial environment that they pass; slowing down cells by bouncing and rolling prior to a tissue-specific molecular interaction (169–171).

LIFESPAN OF MEMORY CELLS AND THE MAINTENANCE OF MEMORY

The idea that memory cells are very long-lived arose as a direct consequence of the observation that memory responses could be invoked months or years after an initial antigenic encounter. It turns out that if you look at some of the original experiments (175, 176), the most you can say is that memory cell clones are long-lived.

Despite this there is still conflicting opinion in the immunological community concerning the lifespan of memory B and T cells. Early experiments by Celada (176, 175) showed that following adoptive transfer memory cells (both B and T helper) decayed in a biphasic manner. Thus, by 30 to 40 days after transfer the "relative memory response" had decreased to 10% of its original level, but further decay (to below 5%) took another 100 days. Celada took no account of whether antigen was transferred with the primed cells; however, Feldbush in similar adoptive transfer experiments noted that the presence of antigen "potentiated" immunological memory (177). At about the same time Askonas and coworkers published data about the serial adoptive transfer of memory B cell clones (identified by isoelectric-focussing pattern of the specific IgG produced in the serum) (178–180). The capacity of the clones to undergo repeated transfer was finite, but transfers usually failed when the recipient was not immunized at the time of injection of cells. These data together with the observed longevity of antibody responses (82, 181) suggest that memory cells may not be long-lived but may require intermittent stimulation from antigen.

In recent years, in my laboratory, adoptive transfer experiments have been performed in which strenuous efforts have been made to transfer memory cells in the absence of antigen (182, 183). We then looked at the

kinetics of decay of these memory cells or the response that they support. The survival of B cells (182), helper and cytotoxic T cells is dependent upon the presence of antigen; in its absence they decay within a matter of weeks (183). These experiments do not allow accurate estimates of physiological half-life, as the cells are transferred into lightly irradiated and therefore reconstituting recipients. However, they do show that antigen plays a role in maintaining all classes of memory cells. The conclusion that memory cells are not long-lived but part of "slowly dividing" clones has not met with universal approval. Schittek & Rajewsky (18) show, in complex BrdU-labelling and cell sorting experiments, that the turnover of memory B cells wanes with time after immunization. At 20 weeks after immunization they find 10% of the antigen-specific memory B cell labelled after an 18 day pulse of BrdU. The conclusion is that memory cells are long-lived with an average lifespan on the order of 6 months. The estimated lifespan may be accurate, but I contend that the turnover of 10% of antigen-specific memory B cells almost half a year after immunization indicates an ongoing, low-level stimulation.

I should point out that none of the published experiments relating to the antigen-dependence of memory cells tells us whether the signal simply allows survival or if it is a stimulus to cell division. There is no reason to believe that a memory B cell differs in its requirements for progression through the cell cycle from any "normal" B cell; T cell signals are probably obligatory (184–186). While there are published data to the contrary (187), we have unpublished experiments that strongly suggest a T cell influence on long-term memory B cell survival, although a cognate interaction may not be necessary.

In humans, some very elegant studies on lymphocyte lifespan have been carried out (188). In the original studies in the 1960s, lymphocytes from patients who had undergone 1500 rad X-irradiation, as treatment for ankylosing spondylitis, were noted to have dicentric chromosomal lesions. For such cells, entry into mitosis leads to cell death, and so their frequency over time gives a measure of the intermitotic lifespan. The number of lymphocytes carrying dicentric chromosomes in the peripheral blood of these patients was assessed over an extended number of years. The decay yielded an estimate of the mean lifespan of a human lymphocyte of 1574 days (4.3 years). A similar study has recently been completed on a similar group of patients, with the benefit of T cell memory markers (CD45RA and CD45RO). CD45RO cells (activated/memory cells) with dicentric lesions disappear within 1 year, in contrast to cells of the CD45RA phenotype, which are still detected 10 years after irradiation (Dr. Peter Beverley, personal communication). The mean half-life of the CD45RA population coincides well with the earlier estimates of the whole T cell population

(188). The shorter half-life of $CD45R^{lo}$ (memory) T cells has been noted previously in sheep (156), while naive cells can survive for long periods after adoptive transfer (189). The model that Beverley and colleagues favor is one in which CD45RO T cells revert to the CD45RA population. It remains to be seen if they still function as memory cells once in this phenotypically naive population. It is also unclear if cells with gross chromosomal lesions can be used to give an accurate measure of the lifespan of normal cells; the lesions might also include damage to genes that are thought to be involved in regulating cell survival. In this context *bcl–2* has been implicated: memory cells derived from *bcl–2* transgenic mice survive for indefinite periods after transfer, in contrast to cells from normal mice (190).

The data I have summarized in this section suggest that memory is a function of continued antigenic stimulation of cells rather than of long-lived memory cells. If antigen is involved in the maintenance of memory B and T cells, then it is of prime importance to identify where antigen might be sequestered for the purpose. A cell that does this has been known for some time (55, 191, 192) and is thought capable of storing antigen for many months (193, 194) and possibly over 1 year (J. Tew, A. Szakal, M. Kosco, unpublished data); this is the follicular dendritic cell (FDC). Clearly the FDC is pivotal for B cell memory and can stimulate memory B cells with the antigen it carries on its surface (195, 196). Their role in maintaining T cell memory is at present obscure, as FDC do not process and present antigen (196) but store antigen-antibody complexes on their surface. For this reason stimulation of memory T cells from this unprocessed antigen depot almost certainly requires the participation of antigen-specific memory B cells as antigen-presenting cells (196). Indeed, if the persistence of T cell memory is influenced by FDC-bound antigen, the logical prediction is that memory T cell development will be dependent on a concomitant antibody response to the antigen, a necessity if antigen is to localize antigen onto the FDC (20, 183). Both of these requirements are under test.

In a normal "wild" population it is probably not necessary to rely solely upon sequestration of a library of antigens to maintain memory. Many viruses persist in the host for extremely long periods in sites often outside the lymphoid system (197, 198). Even if they do not replicate they may remain an antigenic presence. It is also likely that reinfection with common pathogens provides frequent boosts either specifically (infection with the original organism/strain; 197, 198) or cross-reactively (infection with similar or immunologically cross-reactive organisms/strains; 199, 200, 201). Suffice it to say, memory will be long-lived, although probably not completely understood.

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THE ROLE OF AUTOANTIBODIES IN AUTOIMMUNE DISEASE*

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Abstract

In autoimmune diseases, autoantibodies may be the actual pathogenetic agents of the disease, the secondary consequence of tissue damage, or the harmless footprints of an etiologic agent. Establishing a pathogenetic role for autoantibodies requires that they meet stringent criteria. It appears that the location of the presumptive target antigen most critically influences the pathogenetic potential of autoantibodies. Autoantibodies directed against cell surface targets, such as hormone receptors, are clearly pathogenetic; those directed against extracellular targets, such as circulating molecules or extracellular matrix, may or may not cause any damage. Those apparently directed against intracellular targets are usually not pathogenetic unless it can be clearly demonstrated (a) that the antigen is released from within the cell so that it can bind onto a cell surface receptor or other extracellular location, such as proteinase 3; (b) that the antigen moves to an aberrant site on the cell surface, such as, perhaps, the small

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ribonucleoprotein antigen Ro; or (c) that a cross-reactive molecule, the actual target, such as the membrane ribosomal P-like protein, is at an accessible location.

INTRODUCTION

In assessing the role of autoantibodies in autoimmune disease, it is reasonable to remember that, after Ehrlich realized the importance of the possibility that the immune system might react against its host, autoimmune diseases were for decades, and largely remain, those diseases in which autoantibodies are found. At the beginning of the modern era of immunology, the first autoantibodies thoroughly studied—the antibodies of the acquired hemolytic anemias—required no elaborate exegesis. They bound to red cells and sensitized them to lysis by complement or to phagocytosis by the reticuloendothelial system. They caused the clinical diseases in which they were found. But with the discovery of rheumatoid factor and the LE cell factor soon thereafter, autoantibodies without such a clear role were found. Because the diseases with which they associate were (and remain) mysterious as to etiology and pathogenesis, and because autoantibodies seem likely, from the example of hemolytic anemia, to be the agents of damage, a vast amount of research has been devoted to establishing the pathogenetic pathways.

Diseases considered to be autoimmune multiplied, and they came to have a common character: chronic inflammatory illnesses without a known infectious cause in some of whose victims one or another autoantibody is found. Hardly a mysterious disease of this character exists in which an autoantibody reacting with the presumed target organ has not been sought, found, and crowned with a central pathogenetic role.

With the recognition of the cellular immune system as the engine of all immunity including antibody production, there is now a willingness to grant "autoimmune" status to mysterious chronic inflammatory diseases in which are found no autoantibodies and scant evidence of specific cellular autoimmunity beyond lymphocytes infiltrating the affected tissues. Thus the pressure to explain the pathogenesis of "autoimmune" diseases as the product of their autoantibodies has abated, and it is now possible to take a cooler look at the true relationship of these defining elements to the diseases in which they are found.

Autoantibodies may be the actual pathogenetic agents of disease, for example, autoimmune hemolytic anemia; they may arise as a consequence of another disease process, for example, some antibodies that react with nerve tissue or heart muscle after damage to those organs; or they may merely mark, like footprints, the presence of the etiologic agent while not

themselves causing damage. This last role is suspected for many of the best known autoantibodies of human disease.

If autoantibodies are the consequence of tissue damage, one might then find them in individuals who have had organ damage from a variety of unrelated causes. If they are epiphenomena of another etiopathogenetic agent—a virus or toxin—they may nevertheless lead one back to their origin and hence to the origin of the disease. But if they are to be granted status as the true pathogenetic agents of disease, then they must undergo careful scrutiny.

In this review, we set out, as others have before (1–3), criteria for the pathogenicity of autoantibodies, and we measure against them representative examples in an attempt to bring order to a vast literature. We limit ourselves to those autoantibodies associated with human diseases. We do not discuss the fascinating but probably unrelated natural autoantibodies arising primarily from the germline repertoire, and we largely ignore the autoantibodies of nonhuman autoimmune diseases. We do not discuss the autoantibodies associated with malignancies except some of those that are found in paraneoplastic syndromes. We do not discuss cellular immunity nor the role that autoantibodies may have in shaping the T cell repertoire via either idiotypy or other mechanisms. Finally, we by and large evade discussing the events that lead to autoantibody synthesis because that is too large a subject for this review.

We recognize that the target by which an autoantibody is defined is not necessarily the immunogen nor even the actual target with which the antibody reacts in vivo, and we make the assumption, not refuted by any studies we consider wholly believable, that antibodies cannot enter intact cells except through nonspecific mechanisms such as via Fc receptors and, when they do enter by that route, that they cannot reach intracellular contents to cause damage.

CRITERIA OF PATHOGENICITY

Antibodies exert their effects by aggregation, blockade, stimulation, opsonization, complement fixation, sensitization to the actions of other effector cells, direct enzymatic action, or indirect sensitization of a target molecule to other enzymatic action. The number and variety of further consequences of these actions is legion. Although factors such as the class of the autoantibodies, their fine specificity, the valence of both antigen and antibody influence its pathogenetic potential, it is the location of the antigen that is most critical of all. Hence we have divided our analysis according to the location of the putative target and discussed representative examples.

To establish that an autoantibody is pathogenetic, one must be able to

construct a plausible picture of how it might act. The autoantibody should be capable of causing the lesions attributed to it in experimental systems. A suitable immunization that leads to the production of similar autoantibodies should lead to a similar disease process. The antibody should be found along with a plausible target antigen at the site of tissue damage. Autoantibody levels and disease activity should, in general, correlate. Most important and most difficult to demonstrate, removal of the autoantibody should ameliorate the disease process.

Many kinds of observations and experiments have been claimed to support the pathogenetic role for various autoantibodies: in vitro observations of the effects of autoantibodies on molecules, cells, or organs; in vivo observations on disease passed by the transplacental transfer of autoantibodies; passive transfer of autoantibodies to experimental animals; adoptive transfer of autoantibody-producing B cells to SCID mice; the construction of transgenic mice producing autoantibodies; correlation of autoantibody titers with clinical disease manifestations; therapeutic studies of plasmapheresis to remove autoantibodies. In the cases we discuss, we cite the evidence favoring or refuting a pathogenetic role for particular autoantibodies. Few meet the stringent criteria.

To fulfill the requirement of association with a disease, the autoantibody has to be found in patients with the relevant disease in a significantly higher frequency or a significantly higher titer than in normals. The specificity of the antibodies to the disease and not their sensitivity in detecting patients with the disease is the requirement for association. We have also considered the antibody titer in our determination of association because there is often a background response in the normal population, and the pathological response is then defined by a titer several standard deviations above this background. Whether this background binding is of the same nature as that of the disease related antibodies is itself an interesting question, but beyond the scope of this review.

AUTOANTIBODIES TO CELL SURFACES

Antibodies to Intercellular Adhesion Molecules

Pemphigus is a blistering disease of the skin and the mucous membranes characterized by acantholysis—the loss of intercellular adhesion—and by the occurrence of autoantibodies that bind to the surface of epidermal cells. These antibodies however are not just associated with the disease. There is ample evidence that they play a major part in the induction of the clinical lesion.

Direct immunofluorescence of the skin lesions in virtually every patient with pemphigus demonstrates immunoglobulins bound to the epidermal

cell surface. The level of the anti-epidermal antibodies correlates with disease activity and the removal of these antibodies by plasmapheresis leads to clinical improvement (4). The disease can be passively transferred by serum or immunoglobulins derived from patients. For example, incubation of skin or epidermal cell cultures with pemphigus antibodies leads to acantholysis (5); passive transfer of pemphigus immunoglobulins to neonatal mice reproduces the human disease clinically, histologically, and immunologically (6, 7); children of mothers with pemphigus may have transient disease due to maternal antibodies that cross the placenta. Since the predominant anti-epidermal antibody in pemphigus is IgG4 (8–10), and the disease can be transferred to newborn mice by Fab fragments (7), the mere binding of the pathogenic antibodies to a cell surface antigen appears sufficient for acantholysis.

Immunofluorescence studies of skin tissue stained by pemphigus serum demonstrated the presence of autoantibodies directed against the desmosome, a major component involved in epidermal cell cohesion (11). Serum immunoglobulins from patients with the variant, pemphigus foliaceus, bind to a 160 kD glycoprotein that is identical to desmoglein I, a desmosomal core glycoprotein, whereas those from patients with pemphigus vulgaris bind to a 210 kD glycoprotein, synthesized by cultured human keratinocytes. It shows significant homology to members of the cadherin family of calcium-dependent cell adhesion molecules and to desmoglein I (12, 13).

Antibodies To a Neurotransmitter Receptor

Myasthenia gravis is characterized by weakness and fatigability of skeletal muscles and by autoantibodies to the acetylcholine receptor (AChR) (14, 15). These receptors are located at the tips of folds in the postsynaptic membranes of skeletal muscle fibers. They bind acetylcholine released from the nerve ending and, in response, open a cation-specific channel, resulting in local depolarization of the postsynaptic membrane and the triggering of a muscle action potential. Immunization of rabbits with AChR purified from fish electric organs produced anti-AChR autoantibodies and led to severe symptoms resembling myasthenia gravis (16). There is a clear association between the presence of anti-AChR antibodies and the occurrence of myasthenia gravis. Antibodies to human muscle AChR are detected in 85–90% of the patients and are virtually absent in healthy controls (14). Treatment with agents directed at the immune system, such as immunosuppressive drugs, thymectomy, and intravenous immunoglobulins, may lead to clinical improvement and to a decrease in the level of anti-AChR antibody (17, 18).

Other experimental evidence supports a pathogenetic role for auto-

antibodies to AChR. Injection of monoclonal antibodies against the receptors into chickens induces acute myasthenic symptoms within an hour (15). Binding of antibodies to the receptor can presumably directly block the binding of acetylcholine and the proper function of the ion channels. It can also lead to increased receptor degradation. The binding of complement to these antibodies may result in lysis of the membrane (15). Indeed, myasthenic patients have a significant decrease in detectable receptors.

The correlation between the level of antireceptor antibodies and the severity of the disease is not simple. In an individual patient there may be a good correlation between the variation in antibody level and the clinical course, but studies of populations of myasthenia gravis patients show wide variability in the titer of the anti-AChR antibodies without a clear correlation with the severity of muscle weakness. Attempts have been made to improve this correlation by relating the clinical findings not to the total anti-AChR response but to subsets of anti-AChR antibodies. For example, the capacity of myasthenic sera to block the binding of alpha bungarotoxin to human muscle acetyl choline receptors correlated with disease severity (19), and blocking antibodies were found more often in sera of myasthenic mothers who transferred the disease to their infants (20). These observations suggest that at least a subset of the anti-AChR antibodies can be directly pathogenic. However, direct evidence for the pathogenicity of the human antiacetylcholine receptors by adoptive transfer is still missing.

Antibodies To Hormone Receptors

Antibodies against the receptors for pituitary hormones and against the end organ receptors for several major hormones not only have been found but are involved in common and sometimes dramatic diseases. A recent review summarizes work with many endocrine systems (21). Antibodies to receptors for thyroid stimulating hormone and insulin are the best-studied examples, and they are found in relatively common diseases. Their pathogenetic roles have been difficult to sort out because antibodies against the same receptor can stimulate (hormone mimetic) or block the receptor, and in a single patient, both varieties may co-exist. Presumably the exact location of the epitope, the class, valence, affinity, and other characteristics of the antibodies influence their effects.

Antibodies to the peripheral receptor for insulin are found in a small number of cases of insulin-resistant diabetes mellitus or of hypoglycemia (22). Patients who have these rare syndromes usually have an accompanying autoimmune disease, most commonly a lupus-like illness. Since occasional patients with the hyperglycemic, insulin-resistant variant evolve into the hypoglycemic variant with therapy, several theories have arisen

to explain the transition. It has been proposed, for example, that when autoantibodies are in high titer, they lead predominantly to degradation of receptors and thereby resistance to circulating hormone, but when the titer is low, there is only stimulation through a mimetic action of the antibodies. More likely, specificity for different epitopes accounts for the difference in action, and different patients have different mixtures of such variant antibodies.

Assays for the presence of the antibodies use either immunologic methods such as blocking the binding of labelled insulin to cells or immunoprecipitation of affinity-labelled purified receptors, or physiologic ones such as the ability of the antibodies to block the effects of insulin on fat cell metabolism (23). Although the immunologic assays agree with each other, they do not correlate well with the effects on fat cells. Injection of the antibodies may lead to acute hypoglycemia or chronic hyperglycemia in rats (22).

Three major thyroid-related autoantibody specificities have been well-described: to thyroid peroxidase, the so-called microsomal antigen; to thyroglobulin; and to the receptor on thyroid acinar cells for the pituitary thyroid stimulating hormone (TSH). Although antibodies to the first two may correlate with various thyroid and other autoimmune diseases, it is the antibodies to the TSH receptors that most clearly play a role in causing thyroid disease (24). These antibodies were recognized in studies that attempted to identify the factor in the serum of patients with Graves disease, a common form of hyperthyroidism, which might be responsible for both the stimulation of the thyroid gland and the exophthalmos which frequently accompany the disease. The recognition that the so-called long-acting thyroid stimulating substance is immunoglobulin opened this field of study. Autoantibodies in Graves disease are directed against the TSH receptor and mimic the action of the pituitary hormone. Since their production is not subject to the feedback control of the thyroid hormone whose synthesis they stimulate, the gland overproduces the hormone and enlarges under the trophic stimulus delivered through the receptor. The autoantibodies associate strongly with the disease, but they are also found in many cases of the closely related goitrous condition, Hashimoto's thyroiditis, in which the patient may have normal thyroid function at the time the disease is detected but may often become hypothyroid. The precise role of the autoantibodies in these cases is not clear, and the disease is thought to result primarily from the action of the T lymphocytes infiltrating the gland. Much less commonly, antibodies to the TSH receptor may cause hypothyroidism by blocking the action of TSH on the gland (21).

The pathogenetic role of antibodies to the intracellular thyroid peroxidase remains unclear, although there have been attempts, none defini-

tive, to establish that translocation of the peroxidase to the cell surface is an important step in both stimulating the production of autoantibodies and sensitizing thyroid cells to their action (25).

Antibodies To the Surface of Nerve Cells

Antibodies to unique components of nervous tissues have been sought as possible agents of both inflammatory and noninflammatory neurologic diseases. Much attention has been paid to antibodies against gangliosides. These diverse molecules, composed of a lipid, ceramide (the base, sphingosine, attached to a fatty acid), combined with various oligosaccharides, are found in the outer membrane of peripheral nerve cells. IgM antibodies apparently directed at one or another ganglioside can be detected in the sera of patients with several polyneuropathies and diseases of motor neurones (26). However, like many immune assays involving lipids, these assays are tricky to perform, and results are conflicting (27). Two challenges exist to theories that invoke these antibodies as primary pathogenetic agents of disease: One is that they are not disease specific (27–29), and two, that they occur following nervous tissue damage from trauma such as stroke, head trauma (26), or even cervical spondylosis (30). The paraproteins from some monoclonal gammopathy patients with neurologic syndromes have antiganglioside activities, but even these observations merit caution (30). Autoantibodies against α-sulfoglucuronyl paragloboside and sulfatide are elevated in the Guillain-Barré syndrome, but they are not disease-specific (31).

That patients respond favorably to plasmapheresis does suggest that humoral factors play a pathogenetic role. Two large, cooperative trials, both randomized but unfortunately not blinded, purported to show a benefit of early, vigorous plasmapheresis in the Guillain-Barré syndrome (32, 33). The rate of improvement and the long-term outcome were moderately better in those who had plasmapheresis added to a standard regimen. There are conflicting data on whether the improvement correlates with the removal of particular autoantibodies (34, 35).

AUTOANTIBODIES TO EXTRACELLULAR MOLECULES

Anti-phospholipid Syndrome

The association of anti-phospholipid antibodies with repeated episodes of thrombosis, recurrent fetal loss, and thrombocytopenia has been defined as the anti-phospholipid syndrome (36). The anti-phospholipid antibodies include both antibodies against cardiolipin and the so-called lupus anti-

coagulant. The syndrome can be part of SLE or can occur in isolation (37–39).

The main feature of the anti-phospholipid syndrome, thrombosis, may occur in virtually any arterial or venous blood vessel (37). Other manifestations of the syndrome such as thrombocytopenia, transverse myelitis, and chorea are not clearly associated with thromboembolic mechanisms (37). The incidence of recurrent abortions, fetal death, and retardation of intrauterine growth is significant (40).

The frequency of the anti-phospholipid antibodies among lupus patients was found to be much higher than in the normal population (36), and anti-phospholipid-positive lupus patients differ from the anti-phospholipid-negative patients in having a much higher incidence of thrombosis, thrombocytopenia, and fetal loss. For example, thromboses were found in 42% of lupus patients with anti-phospholipid antibodies but in only 13% of those without the antibodies. This strong association between anti-phospholipid antibodies and thromboembolic events is not restricted to known autoimmune conditions. The prevalence of anti-phospholipid antibodies in young patients with stroke was found to be around 45%, similar to their prevalence in SLE patients with thrombosis (41). These data provide firm evidence for the association between anti-phospholipid antibodies and clinical evidence of thromboembolism, but are these antibodies pathogenic?

Their pathogenetic role in the induction of recurrent abortions has been studied recently by the use of a passive transfer model. Transfer of antibodies derived from a patient with primary anti-phospholipid syndrome to mice increased the resorption index of embryos, lowered the number of embryos per pregnancy, and decreased the weight of embryos and placenta (42), findings that simulate some properties of the human disease.

Both anti-cardiolipin antibodies and the anticoagulant antibodies found in lupus appear to bind anionic phospholipids, but they recognize different structures. The conventional paradigm that anti-cardiolipin antibodies bind directly to the phospholipids has recently been challenged by the discovery that the binding of these antibodies to the phospholipids requires the presence of a serum cofactor (36, 43, 44). This cofactor has been identified as the plasma glycoprotein, β2-glycoprotein-I. The antibodies bind neither to β2-glycoprotein-I nor to cardiolipin alone. It seems, therefore, that they are directed against a complex consisting of β2-glycoprotein-I bound to anionic phospholipid or a cryptic epitope formed during the interaction of the two molecules.

The identification of β2-glycoprotein-I as the target antigen for the anti-cardiolipin antibodies may help in clarifying the mechanism by which these

antibodies can lead to increased coagulability. β2-glycoprotein-I has been found to interact with various steps of the coagulation pathways. It binds to platelets, inhibits ADP-mediated platelet aggregation, and inhibits the intrinsic coagulation pathway. Interference with these activities might therefore lead to hypercoagulation.

Antibodies to a Circulating Hormone—Insulin

Autoantibodies to insulin are most often found in diabetics who repeatedly inject themselves with insulin, but sometimes these occur spontaneously and may present as hypoglycemia. The mechanism of this paradoxical phenomenon is thought to be either antibody-induced potentiation of insulin's action or a complex interplay of circulating free and antibody-bound hormone. Although it is tempting to speculate that antibodies to the hormone itself or to the hormone receptor arise as an idiotype-anti-idiotype system, this has usually not proved to be the case (23).

Antibodies to an Extracellular Protein—Type II Collagen

Arthritis may be induced, at least in some species and under some conditions, by either active or passive immunization with the dominant structural protein of cartilage, type II collagen (45). It seems likely, however, that such antibodies in humans and other species with spontaneous arthritis are not disease specific but arise nonspecifically in response to joint damage (46). Nevertheless, they may have a secondary role in pathogenesis.

AUTOANTIBODIES TO INTRACELLULAR MOLECULES

Understanding the relationship between antibodies to intracellular antigens and the diseases in which they are found is challenging; the burden of proving that they are pathogenetically important is considerable; and for many of the systems studied, there is too little information for serious analysis. Furthermore, little direct experimental testing has been done of proposed models.

Antimitochondrial Antibodies

Anti-mitochondrial antibodies have an extremely strong association with the chronic liver disease, primary biliary cirrhosis (47). In this disease, T cells infiltrate and destroy the smallest intrahepatic bile ducts, leading to slowly progressive biliary obstruction, jaundice, and ultimately cirrhosis. Essentially all patients with this disease, and almost no others, have anti-

mitochondrial autoantibodies. Bile duct damage from identifiable extraneous causes does not lead to anti-mitochondrial autoantibodies.

Although the antibodies do not discriminate among mitochondria from different tissues, there is little pathology elsewhere, except that a proportion of patients have similar damage to their small salivary ducts. The principal antigenic target is the inner lipoic acid–binding domain in dihydrolipoyl acetyltransferase, one of the three peptide components of the multienzyme complex pyruvate dehydrogenase, which is found in the inner mitochondrial membrane (48). Thus, the target antigen is not only common to all cells with mitochondria but is separated from the plasma by two membrane systems, making passage of the antibodies to this intracellular target an unlikely event.

Several observations confirm the unlikeliness that the antibodies per se cause the illness. If a normal liver is transplanted into a patient with primary biliary cirrhosis, the disease does not recur despite the continuing presence of anti-mitochondrial antibodies (49). When mice, rats, guinea pigs, rabbits, and rhesus monkeys were immunized with the recombinant human enzyme, all the animals made antibodies that inhibited the overall function of the pyruvate dehydrogenase complex, and the antibodies from both rabbits and guinea pigs specifically reacted with the functional region of the acetyltransferase portion (50). All the antisera reacted with the human protein used for immunization, but some did not react with their own mitochondria. Despite the similarities to the spontaneous antibodies found in the human disease, there were differences in the fine specificity as judged by cross-reaction with other components of the multienzyme complex. Although the antibodies were made in high titer, none of the animals showed histological or chemical evidence of liver damage.

An attempt to produce the illness in SCID mice by injecting them with peripheral blood lymphocytes from primary biliary cirrhosis and control patients gave equivocal results (51). Anti-mitochondrial antibodies were produced, and some animals showed bile duct damage, but bile duct damage was also part of what appeared to be a graft-vs-host reaction of SCID mice that received normal lymphocytes and that never had anti-mitochondrial antibodies. It was impossible to assess the role of the antibodies or even of the B cells because unseparated cells were transferred.

If the autoantibody response to the target protein results from a dislocation of the antigen from a privileged intracellular site because of a primary pathogenetic event in bile ducts, the same event might render the damaged ducts susceptible to damage by the autoantibodies. There is, however, no clear evidence for such a dislocation, and if it did occur as the result of some primary pathogenetic event, finding a special pathogenetic role for these disease-specific autoantibodies would be difficult to discern.

Antibodies to Cytoplasmic Enzymes: Anti-aminoacyl-tRNA Synthetases in Myositis

The autoantibodies associated with idiopathic, presumably autoimmune, inflammatory muscle disease (myositis) have been intensively studied in many laboratories including our own. Patients with myositis often have one or another of an interesting group of disease-specific autoantibodies. These antibodies are mostly directed at proteins or ribonucleoprotein particles that are concerned with protein synthesis. Most have bound RNA, and most are cytoplasmic. These autoantibodies are not only limited to patients with myositis; each is associated with a distinct clinical syndrome, a syndrome that tends to occur preferentially in patients with a particular histocompatibility type. The syndromes are distinguished by physical findings, severity, rapidity of onset, response to therapy, and even, on occasion, season of onset (52, 53).

Most intensively studied are the patients with antibodies to histidyl-tRNA synthetase or to one of four other aminoacyl-tRNA synthetases (glycyl-, alanyl-, threonyl-, or isoleucyl-). These autoantibodies have been found essentially in no other disease. They bind the relevant enzyme (54), usually with tRNA bound, or sometimes the cognate tRNA in addition (55), and they inhibit the action of the enzyme. The enzymes have not been found on the surface of myocytes or of any other cell, but at least in the one case for which good information is available, there is strong reason to implicate intact histidyl-tRNA synthetase as the driving antigen for the autoantibody synthesis (56). The titer of the autoantibody fluctuates with disease activity and may even disappear when the disease is in remission (57). Of considerable interest are studies in one patient from whom serum specimens were obtained at a time when the patient clearly had neither clinical nor laboratory evidence of myositis. The antibody response began several months before the disease was clinically or chemically evident, passed through a stage demonstrating affinity maturation against the enzyme, and was a fully developed response of broad spectrotype by the time the patient became sick with myositis. However, in a recent controlled trial, plasmapheresis did not benefit patients with autoimmune myositis (58).

It seemed possible that the autoantibodies arose as a result of infection with a myotropic picornavirus such as encephalomyocarditisvirus, because these viruses can cause myositis, and picornavial RNA may serve as a substrate for aminoacyl-tRNA synthetases (59, 60). However, mice infected with encephalomyocarditisvirus did not develop autoantibodies to synthetases (61), and a series of extremely sensitive assays for several candidate viruses in the muscles of patients with myositis turned up no

evidence of persistent picornaviruses (62). These autoantibodies are thus most usefully considered as an intimately linked phenomenon of doubtful pathogenetic role in this syndrome.

Antibodies to Other Intracellular Components

Autoantibodies to cardiac myosin are an excellent example of an autoantibody to an intracellular component that arises in response to damage to an organ. A wide variety of insults to the heart, including myocarditis, myocardial infarction, valve surgery, and coronary artery bypass grafting are followed by the appearance of anti-myosin antibodies (63). There is no consistent pattern of immunologic injury that can be attributed to these antibodies. Experimental mouse myocarditis induced by a picornaviral infection also led to antibodies to cardiac myosin, but cardiac damage could not be ascribed to the antibodies: they localized only to already damaged cells in vitro; they did not localize to myocytes and caused no illness when passively infused in vivo; and the myocarditis occurred even when autoantibody production was blocked (64).

There is a vast array of autoantibodies to nuclear components, and many have been closely studied (65). Among well-studied or recent examples are the antibodies associated with scleroderma (66, 67), and autoantibodies to the mRNA splicing machinery (68, 69), autoantibodies to an 80-kDa nuclear component only clearly visible in interphase nuclei (70). What is lacking is almost any evidence connecting the autoantibodies to a plausible pathogenetic role. There is scant and unconvincing evidence that the antigens themselves are ever exposed as a target except, presumably, when they are released during gross cell lysis; nor do the fluctuations with disease activity or in response to effective therapy strongly point to a pathogenetic role.

AUTOANTIBODIES TO INTRACELLULAR COMPONENTS THAT APPEAR ON CELL SURFACES OR IN THE EXTRACELLULAR ENVIRONMENT

Antibodies To Ribonucleoproteins

An autoantibody system for which there is considerable useful information is the anti-ribosomal system, largely limited to patients with lupus. These autoantibodies react with the P proteins of the ribosomes, and they occur in both spontaneous human lupus and spontaneous murine lupus in the MRL mouse strain. At least in mice, it seems highly likely that the continued production of the antibodies is driven by autologous ribosomes (71). Human patients who have these autoantibodies often have, in addition, autoantibodies that bind to the portion of the RNA that combines with the P proteins to form a GTPase enzymatic activity (72). This resembles

several other systems in which there are antibodies to both the protein and the RNA of a ribonuclear protein particle. The anti-ribosomal P protein antibodies have an intriguing clinical association in several studies: they are found most often (73, 74), though not exclusively, in patients with depression or psychosis. There is some evidence that disease activity and antibody levels are connected (74, 75). The antibodies to the RNA component and to the protein component appear to fluctuate independently, not as though they constituted an idiotype-anti-idiotype system (72).

New observations on the reaction of affinity-purified anti-ribosomal P protein autoantibodies with cells have opened up the fascinating possibility that these antibodies exert a pathogenetic effect by cross-reacting with a protein on the external membrane of cells (76). Not only do the affinity-purified antibodies bind to the surface of intact cells, as determined by careful fluorescent and electron microscopic observations, they also bind specifically to a protein of the same size as the previously identified target from ribosomes when tested by Western blotting with a plasma membrane preparation. Thus, although much prior evidence suggests that these autoantibodies have had their production driven by their presumed target, ribosomes, they may become pathogenetically significant only by virtue of a cross-reaction or the previously unrecognized presence of a ribosomal protein on cell membranes.

Another example of a similar phenomenon may be the autoantibodies against the intracellular small ribonucleoprotein particles, Ro(SS-A) and La(SS-B) which frequently are associated with SLE, Sjogren's Syndrome, and the apparently transplacentally mediated syndromes, neonatal lupus and congenital heart block. These syndromes occur in a small proportion of the infants born to women with circulating anti-Ro, anti-La, and anti-RNP autoantibodies. The predominant evidence links the cardiac syndrome to anti-Ro and anti-La autoantibodies (these antibodies are uncommonly found separately) (77). Constructing a plausible pathogenetic pathway has led investigators to seek the presence of the autoantigens, especially Ro, on sites accessible to the antibodies delivered transplacentally. It would be particularly satisfying to discover them on the surface of fetal but not adult cardiac conduction tissue. A recent study, which reviews the experimental evidence, showed that immunoglobulins containing anti-Ro autoantibodies from the mothers of children with congenital heart block and from the mothers of children without heart block bound fetal but not adult cardiac tissue and altered transmembrane action potentials in ex vivo preps (78). Sera with other SLE-related specificities did not produce these effects. A fully satisfactory pathogenetic story, however, is not yet apparent. The actual identity of the autoantibody specificity and the exposed antigen remain to be found in order to account for the sporadic and low incidence of the congenital syndromes.

Antibodies To an Intracellular Lysosomal Enzyme Which May Be Secreted

A family of autoantibodies that seems to have the potential to play at least an amplifying role in the pathogenesis of an idiopathic inflammatory illness is the group known as ANCA, anti-neutrophilic cytoplasmic antibodies (79). The most specific of these, c-ANCA, is directed against a 29-kDa serine protease (proteinase 3) which is a component of the primary lysosomes of neutrophils and monocytes. In neutrophils, the enzyme is found in the azurophilic granules released with activation of the cell. This antibody is found essentially only in the granulomatous vasculitis known as Wegener's granulomatosis, in a related group of kidney diseases, crescentic or necrotizing glomerulonephritis, and less commonly in other vasculitides. Not only is the disease association strong, but a rise in titer of this antibody precedes most clinical flares of the disease (though not all rises in titer are accompanied by a flare). The antibodies are not commonly seen in any other illnesses with a vasculitic component and are therefore unlikely to result simply from vascular injury (80).

In vitro experiments with neutrophils can be made to show a pathogenetic effect of c-ANCA IgG. If the IgG is added to neutrophils "primed" by various cytokines, especially TNF, the neutrophils are "activated" as shown (i) by a rise in superoxide radical formation, (ii) by changes in protein kinase C and other second messenger pathway components, and, most relevant, (iii) by granule release (81). Some evidence suggests that the reaction called priming, which is nonspecific and difficult to control in neutrophils ex vivo, brings the components of the granules to the cell surface. Theoretically, this might translocate the normally intracellular antigen to a site where circulating antibodies could bind to it. It has been proposed that the exposed antigen is the primary target, and that the encounter with antibody brings the cells from the primed state to the fully activated state, and to release of much more granular material into the medium. This could lead to the local formation of immune complexes, complement fixation, and a widening inflammatory cascade with a built in vicious cycle.

It is possible, however, that the priming state itself simply releases the granular contents rather than translocating them to the membrane, and that all the subsequent events are due to immune complex activation of neutrophils, a long-studied phenomenon. Probably the most critical event following neutrophil aggregation is adherence of the neutrophils to vascular endothelium where the released granular material may directly damage cell walls. Thus, the accelerating role of a circulating antibody to a granular component is not hard to imagine. In summary, this may be a

system in which an autoantibody to an intracellular component exerts a pathogenetic role because the component is released into the extracellular space in a location where the consequences of the local formation of immune complexes are likely to be severe.

Anticerebellar Autoantibodies in Cancer

The subject of the antibodies against neoplastic tissue that appear in patients with tumors is vast and largely beyond the scope of this review. However, there are some so-called paraneoplastic syndromes in which a clinical syndrome that can be directly attributed neither to the mass of the primary tumor nor to metastases appears to be due to autoantibodies. A well-studied set of tumor-associated neurologic syndromes appears to result from antibodies that react both with the tumor and with cells of the central or peripheral nervous systems.

For example, patients with breast or ovarian cancer may develop cerebellar abnormalities as the first manifestation of their tumor (82, 83). Indeed, in these patients, the neurologic findings often lead to the diagnosis of the tumor. Post-mortem examination shows degeneration of the Purkinje cells of the cerebellum, and several groups have documented that the serum contains antibodies that react specifically with the cytoplasm of Purkinje cells (84, 85). The antibodies react with a 62-kDa protein or, to a lesser extent, with a 35-kDa protein. The antibodies, when purified on cerebellar extracts, react with sections of tumors from patients who have the antibodies, but not from similar tumors from patients who lack the antibodies. Tumor extracts from patients with the antibodies, but not from those without the antibodies, contain the 62-kDa Purkinje antigen (85). The simplest explanation is that some tumors abnormally produce the cerebellar antigen that is targeted by the immune system because it has lost its immunologically privileged position in the central nervous system. More difficult to explain is how the antibody damages the Purkinje cells, since the antigen appears on immunofluorescence to be cytoplasmic. A different autoantibody has been found in a patient with cerebellar degeneration without a tumor. It reacts with several other Purkinje cell proteins and is interesting because it also reacts with some tumors of supposed neuroectodermal origin: melanoma, small cell lung carcinoma, and neuroblastoma (85). Until these antigens are better characterized and their cellular locations are known in more detail, it is not possible to construct a fully plausible pathogenetic picture of the events leading to the syndrome.

Antibodies to DNA and Histones

Although low titers of anti-DNA autoantibodies may occur in various inflammatory and autoimmune diseases, high levels are found mainly in

systemic lupus erythematosus (SLE), and the combination of a high antibody to double-stranded DNA with low complement levels is virtually diagnostic of SLE (86). But are these antibodies pathogenic? If they are, what are the target antigens in vivo? Are they cross-reacting molecules such as sulfated glycolipids (87) or glycosaminoglycans (88)? Are they circulating DNA molecules liberated from dead cells into the circulation where they form immune complexes? Or are they DNA, histones, or intact nucleosomes that have reached and bound to the surface of intact cells by specific receptors, as recent evidence suggests (89–93)?

The experimental support for direct pathogenetic effects of anti-DNA antibodies has come from several sources. The capacity of lupus antibodies to bind directly to the kidneys has been studied by perfusion experiments. Perfusion of kidneys with certain monoclonal anti-DNA antibodies led to deposition of immunoglobulin and to modification of kidney function, and anti-DNA antibodies from lupus patients with active nephritis bind to glomeruli in an in vitro perfusion model and also induce proteinuria (94). The nature of the glomerular target antigen is still not clear, but candidate molecules include the laminin and the heparan sulfate components of the glomerular basement membrane which cross-react with anti-DNA antibodies (88).

Historically, lupus was considered to be the prototype of a spontaneous immune-complex disease in which anti-DNA antibodies form immune-complexes with circulating DNA and deposit in tissues (e.g. glomeruli) where local complement activation leads to an inflammatory response. In fact, anti-DNA autoantibodies could be eluted from the kidneys of both humans (95) and mice (96) with lupus nephritis. The antibodies that are isolated from the lesion may differ in their specificity from the antibodies found in the circulation. For example, anti-DNA antibodies eluted from the kidneys of a lupus patient or from MRL-lpr/lpr mice cross-reacted with sulfated glycosaminoglycans, whereas serum anti-DNA antibodies did not (97), and other differences in the ligand binding properties of kidney eluate anti-DNA antibodies from lupus patients and their serum counterpart have been noted (98). These findings suggest that the kidney binding autoantibodies in lupus may be a subpopulation of anti-DNA antibodies characterized by specificity for certain antigens as glycosaminoglycans.

The mechanisms of immune-complex deposition have been thought to follow the paradigm of induced immune complex disease, serum sickness, in which the site of deposition of the complexes is governed mainly by their physical characteristics. More recently, it has been suggested that the deposition of DNA-anti-DNA complexes in the tissues is not due to passive, nonspecific trapping. Instead it may result from a specific inter-

action between the antigenic component of the complexes, namely DNA, and tissue bound histones (88, 99, 100), because histones can bind to the heparan sulfate constituent of the glomerular basement membrane (101). These cationic molecules could target the complexes toward the kidney. Indeed, perfusion of kidneys with histones, DNA, and anti-DNA antibodies resulted in the glomerular deposition of immune-complexes, suggesting that the binding of the DNA-anti-DNA to the glomeruli is mediated via the histones (102). Renal biopsies from lupus patients stained with anti-heparan sulfate antibodies showed the disappearance of the normal glomerular heparan sulfate, possibly due to the masking of the negatively charged polysaccharide by positively charged histones (103). Similarly, the binding of a lupus anti-DNA antibody to a 94-kDa cell-surface protein is apparently mediated via a DNA-histone complex (99).

In a study that attempted to demonstrate that the entire syndrome of lupus in mice could follow from the presence of anti-DNA autoantibodies, a human monoclonal antibody carrying a major lupus idiotype 16/6 was reported to induce serological as well as pathological changes typical of lupus when administered to non-autoimmune mice. The dose of the antibody used was small, and it did not seem to interact directly with the target organs. Presumably it elicited an anti-idiotypic response which in turn induced murine autoantibodies with the same idiotype. Immunohistological studies of the mouse kidneys indeed revealed deposition of mouse antibodies carrying the 16/6 idiotype (104). These experiments have not been reproduced convincingly by others (105).

SCID mice that received lymphocytes from human lupus patients produced antinuclear antibodies (106, 107). Although in some of these mice, deposition of IgG and complement was detected in the kidney, this was not associated with the histological findings of glomerulonephritis (106). The introduction of an anti-DNA transgene to non-autoimmune mice also failed to answer the question of pathogenesis because the mice developed tolerance to the autoimmune cells and did not secrete anti-DNA antibodies (108).

Although there is also a reasonably good relationship between the level of these antibodies and disease activity, the addition of treatment with plasmapheresis does not improve the clinical outcome in patients with SLE and severe nephritis as compared with use of the standard regimen alone (109, 110).

Some evidence suggests that cytoplasmic or nuclear antigens can be displaced to the cells surface where, it is supposed, they can serve as the target of autoantibodies. Thus, estrogens (111, 112), ultraviolet light (113, 114), and certain tissue culture perturbations (115) have been shown to

bring certain target autoantigens such as Ro, La, or RNP to the cell surface, as determined with monospecific patient sera. However, the cell surface target molecules have not yet been rigorously identified.

Autoantibodies to a Target That May Be Both Cytoplasmic and Cell Surface

In insulin-dependent diabetes, insulin-secreting beta cells of the pancreas are destroyed, probably as the result of an autoimmune attack that begins several years before the disease becomes manifest (116). During this premonitory period, the serum of many prediabetics contains autoantibodies directed at islet cells and their products (117–119). The earliest antibodies to appear are those directed at a 64-kDa islet cell antigen (120). In longitudinal studies of people at risk for diabetes—presumed pre-diabetics on the basis of family studies—the occurrence of these autoantibodies may predict subsequent disease, although the data are not completely convincing (121–123). When the disease does appear, the autoantibodies may disappear. Similar antibodies were found in diabetic BB rats and NOD mice. In the spontaneously diabetic BB rat, antibodies to a rat islet cell 64-kDa protein were found between 12 to 22 days of age, whereas the disease develops about 50 days later (124).

It was the discovery of a patient with diabetes, epilepsy, and a rare neurologic illness—the stiff man syndrome, which is characterized by muscle spasms and antibodies to those neurones that produce gamma-aminobutyric acid—that led to a fascinating set of observations. First, the 64-kDa diabetes-associated target antigen and the target antigen in this subset of neurones are the same protein: glutamic acid decarboxylase. This enzyme is found in pancreatic beta cells, where its function is still unknown, and in those neurones, where it is the final enzyme in the synthetic pathway that produces the inhibitory neurotransmitter, gamma-aminobutyric acid. Second, there is evidence that this presumed cytoplasmic enzyme is also present on the plasma membranes, thus explaining how it is exposed to the immune system and how it may serve as a target for autoantibodies (125). Third, there is a highly suggestive homology between a 24-amino-acid sequence in glutamate decarboxylase and the P2-C protein of a picornavirus, Coxsackievirus B4, which has been otherwise implicated in the etiology of diabetes mellitus (123). Thus, an etiologic agent, a cross-reactive self-antigen, a possible site of encounter between the self antigen and the immune system, and epidemiologic evidence connecting the etiologic agents and the autoantibodies are all present. Furthermore, plasmapheresis may ameliorate the stiff man syndrome (126). However, a direct effect of the autoantibodies has not yet been demonstrated.

UNUSUAL ACTIONS OF AUTOANTIBODIES

A peculiar phenomenon has recently been studied with several naturally occurring autoantibodies isolated from patients. Autoantibodies to the vasoactive intestinal peptide apparently cleave the peptide at several sites within one region of the molecule (127). The exposure of this region rather than the particular amino acid sequence seems important. Unrelated peptides were not cleaved, but very few substrates have been tested. There have been no studies with induced antibodies to the same peptide, nor have any monoclonal or hybridoma antibodies been studied. If this phenomenon is borne out in further studies and is found with other naturally occurring autoantibodies, it will be interesting to identify physiologic consequences of the proteolysis.

In similar but even more preliminary studies, antibodies that hydrolyze DNA have been identified in the serum of a lupus patient with anti-DNA autoantibodies (128).

Finally, binding of autoantibodies to C1 inhibitor renders it susceptible to proteolysis and inactivation by the very proteases it normally inhibits (129). The release of these proteases from their normal inhibition allows unchecked complement activation and the proteolytic release of peptides responsible for the clinical syndrome of angioedema.

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DEFENSINS: Antimicrobial and Cytotoxic Peptides of Mammalian Cells

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Abstract

Defensins are antimicrobial and cytotoxic peptides that contain 29–35 amino acid residues, including six invariant cysteines whose intromolecular disulfide bonds cyclize and stabilize them in a complexly folded, triple-stranded β-sheet configuration. Generated by the proteolytic processing of 93–95 amino acid precursor peptides, they constitute $>5\%$ of the total cellular protein in human and rabbit neutrophils (polymorphonucleated neutrophils—PMN) and are also produced by rabbit lung macrophages and by mouse and rabbit small intestinal Paneth cells. Despite their prominence in rat PMN, defensins are not found in murine PMN. The antimicrobial spectrum of defensins includes gram positive and gram negative bacteria, mycobacteria, *T. pallidum*, many fungi, and some enveloped

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viruses. Defensins exert nonspecific cytotoxic activity against a wide range of normal and malignant targets, including cells resistant to TNF-α and NK-cytolytic factor. They appear to kill mammalian target cells and microorganisms by a common mechanism, which involves initial electrostatic interactions with negatively charged target cell surface molecules (likely the head groups of polar membrane lipids), followed by insertion into the cell membranes which they permeabilize, forming voltage-regulated channels. In addition to their antimicrobial and cytotoxic properties, some defensins act as opsonins, while others inhibit protein kinase C, bind specifically to the ACTH receptor and block steroidogenesis or act as selective chemoattractants for monocytes. Defensins are a newly delineated family of effector molecules whose contribution to host defense, inflammation, and cytotoxicity may be considerable for humans, even though it is unlikely to be revealed by experimentation with mice.

INTRODUCTION

Polymorphonucleated neutrophils (PMN), the most abundant leukocytes in circulating blood, normally outnumber its other phagocytes, monocytes and eosinophils, by 10 to 20 fold. All of these phagocytes can kill microorganisms or eukaryotic target cells by exposing them to toxic oxidants or to peptides and proteins that act via nonoxidative mechanisms. Most of the phagocyte's nonoxidative effector molecules are stored within membrane-bounded cytoplasmic organelles ("granules") whose contents are delivered to intracellular vacuoles or to the cell's surface by a secretory process called "degranulation."

Defensins are a group of homologous peptides that may equip certain mammalian neutrophils, macrophages, or small intestinal Paneth cells to damage a wide variety of prokaryotic and eukaryotic targets. These peptides were first recognized in mammalian PMN around 30 years ago, when Zeya & Spitznagel reported the presence of "lysosomal cationic proteins" with antimicrobial properties in rabbit and guinea pig PMN (1, 2). We began to explore this area over a decade ago, while examining the nonoxidative fungicidal and cytotoxic mechanisms of mammalian macrophages (3, 4) and granulocytes (5, 6).

Having initially purified two potently microbicidal peptides, MCP–1 and –2, from rabbit lung macrophages (7), we purified six more peptides (NP–1, –2, –3a, –3b, –4 and –5) from rabbit PMN. When all had been sequenced, we found that they were homologous and that MCP–1 and –2 were identical to NP–1 and –2, respectively. We first used the term "defensin" in describing HNP–1, –2, and –3, the human homologs of these rabbit peptides and later used it to encompass homologous molecules found in

other species. Most recently, other antimicrobial peptides that contain six cysteine residues and three intramolecular disulfide bonds (e.g. "insect defensins") have also been called defensins, although they are not homologous (8) to the peptides discussed here.

BIOCHEMICAL FEATURES

Purification

Purification of defensins from human (9), rabbit (10), guinea pig (11, 12) and rat neutrophils (13) has typically been accomplished by a combination of techniques, including size exclusion, ion exchange, and hydrophilic interaction chromatography, preparative gel electrophoresis and reversed phase high performance liquid chromatography. Because some defensins (e.g. rabbit NP–1 and NP–2) lack tyrosine or tryptophan residues, such columns are routinely monitored at 225–235 nm and 280 nm. Anomalous (most often delayed) elution of some human and rabbit defensins from "sizing" columns packed with several gel matrices, including Sephadex, Biogel, and Sephacryl (14, 15), is not unusual. A combination of preparative electrophoresis and reversed phase high performance liquid chromatography has proven especially useful for purifying defensins from very small amounts of starting material. Ultra sensitive antimicrobial assays that detect nanogram quantities of defensins are available for such settings (16). Several laboratories have produced defensins by solid phase chemical synthesis, and some of these have recently become available commercially, although not yet inexpensively.

Primary Sequences

Mature (i.e. processed) defensins contain 29–35 amino acid residues, including six invariantly conserved cysteines which form three intramolecular disulfide bonds (12, 17–23). They are invariably cationic, and the disulfide bond connecting their amino-terminal and carboxy-terminal cysteine residues renders them effectively cyclic (20). These intramolecular cysteine disulfide bonds are also essential for antimicrobial and cytotoxic activity. Figure 1 shows the primary amino acid sequences of many currently described mature defensins. The conserved residues that form the characteristic defensin motif are boxed. Note that these include all of the cysteines.

Three-Dimensional Structures

The three-dimensional structures of several defensins have been analyzed by nuclear magnetic resonance spectroscopy (24–26) and by X-ray crystallography (27). Both techniques showed that defensin molecules consisted of structurally rigid β-sheets stabilized by their intramolecular di-

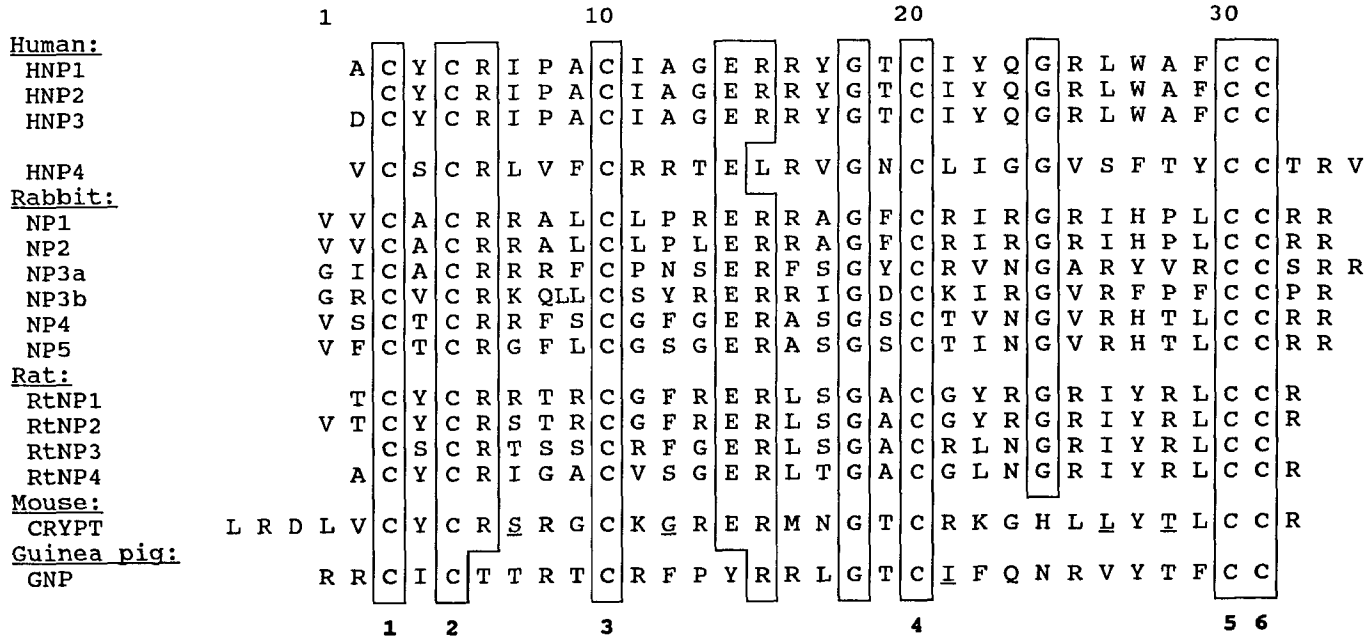

Figure 1 Amino acid sequences of the known mammalian defensins. Amino acids are numbered at the top based on the sequences of the rabbit peptides. Boxes are placed around the highly conserved amino acids, including the six cysteines numbered 1 through 6 at the bottom. The disulfide linkage of these cysteines for HNP–3 has been established as 1–6, 2–4, 3–5.

sulfide bonds. No α-helical domains were present. The 2-D NMR studies of NP–5 (26) confirmed that a disulfide bond connected cysteine 1 (amino-terminal) and 6 (carboxy-terminal) and apposed the two ends of the molecule. They also indicated that the disulfide bond between cysteines 2 and 4 connected one β-hairpin strand to the peptide's N-terminal region while the bond between cysteines 3 and 5 connected the other hairpin strand to its midsection. X-ray crystallography revealed that the human defensin HNP–3 was an elongated ellipsoidal molecule, 26 × 15 × 15 Angstroms, whose structure was dominated by a three-stranded, cystine-stabilized antiparallel β-sheet (27). The highly conserved Arg^6 and Glu^{14} residues formed a salt bridge spanning the only non β-sheet portion of the molecule and the invariant $glycine^{24}$ occupied the third position of a Type I′ turn. HNP–3 crystallized as a dimer that configured a six-stranded β-sheet stabilized by hydrophobic interactions and hydrogen bonds. The invariant $glycine^{18}$ was located at the dimer interface, suggesting that dimer formation may be an essential element of defensin-mediated activity. Defensin dimers were shaped like a twisted and coiled basket that had an apolar base and a polar top. The amino-terminal β-strands of each defensin monomer were hydrogen bonded together through ordered solvent molecules that formed a mini-channel, which traversed the dimer. Based on these structural features, several models were proposed for defensin-mediated cytotoxic and antimicrobial activity (27). Common to each of them was an initial phase of electrostatic binding between the defensin's arginine groups and the negatively charged head groups of the target cell membranes, which concentrated defensins at the surface of the outer leaflet. From this site, defensins could be pulled into the target cell membrane by the negative electrical charge of the inner leaflet. Both dimer formation and a facilitating role for transmembrane potential were consistent with the observed effects of defensins on artificial lipid bilayers (28) and on prokaryotic and eukaryotic targets, discussed below.

ANTIMICROBIAL ACTIVITY

Gram-Positive and Gram-Negative Bacteria

When tested in vitro at concentrations between 10 and 100 μg/ml, purified defensins killed a wide variety of bacteria (Table 1). In general, they exhibited greater potency against gram-positive bacteria (and fungi) than against gram-negative bacteria. Defensin-mediated antibacterial activity was enhanced in low ionic strength media that contained microbial nutrients but lacked significant concentrations of Ca^{2+} or Mg^{2+}. Very arginine-rich defensins, such as rabbit NP–1 or 2 or RatNP–1 were usually five to ten times more potent than human HNP–1 in vitro against most bacteria.

Table 1 Defensin-sensitive bacteria

	Active denfensins	Reference
Gram positive bacteria		
Staphylococcus aureus	NP-1,2,3a,3b; RatNP-1,3,4; GPNP-1,2, HNP-1,2	11, 13, 98
Staphylococcus epidermidis	NP-1,2,3a,3b	10
Streptococcus agalactiae	NP-1,2,3a,3b	10
Streptococcus pneumoniae, Type III	NP-1,2,3a,3b,4,5	10
Streptococcus faecalis	NP-1,2	7
Listeria monocytogenes	NP-1,2,3b	7, 10
Bacillus subtillis	NP-1,2	7
Gram negative bacteria		
Escherichia coli	NP-1,2; HNP-1,2,3,4	7, 98
Salmonella typhimurium	NP-1,2	7, 44
Serratia marcescens	NP-1,2,3a,3b	10
Acinetobacter calcoaceticus	RatNP-1,3,4	13, 99
Haemophilus influenzae, Type 3a	NP-1,2,3a,3b,4,5	10
Pseudomonas aeruginosa	NP-1,2,3a,3b; HNP-1,2	10, 98
Capnocytophaga spp.	HNP-1,2,3	100
Actinobacillus actinomycetemcomitans	NP-1	101

Fungi

The ability of rabbit and guinea pig "lysosomal cationic proteins" (defensins) to kill *Candida* spp. and *Cryptococcus neoformans* (1, 5, 6) was established well before their primary structures had been delineated. NP–1 and NP–2, rabbit defensins expressed both by PMN and alveolar (lung) macrophages, were highly effective against *C. albicans* (29). Their candidacidal effects began within minutes and were associated with a prompt cessation of oxygen consumption and rapid loss of intracellular Rb^{86}, an analog of potassium (14). Candidacidal activity could be inhibited by increasing the ionic strength or the Ca^{2+} concentration of the assay medium or by performing the study at 0°C.

Binding of NP–1 to *C. albicans* was biphasic (30). Its initial phase was reversible, saturable, and of high capacity, accommodating $>5 \times 10^7$ defensin molecules per blastospore. It was significantly reduced in high ionic strength media but not by addition of Ca^{2+} or by conducting the incubations at 0°C. The second phase of binding occurred only under conditions that supported candidacidal activity, and was inhibited by mM concentrations of Ca^{2+} but not Mg^{2+}. NP–1 binding was competitively inhibited by NP–2 or NP–3a but was unaffected by NP–4 or NP–5. Although NP–5 had little intrinsic candidacidal activity, it nevertheless potentiated the candidacidal effects of low concentrations of NP–1 (31).

Human defensins HNP–1 and –2 were effective against *C. albicans*, whereas their close congener, HNP–3 was inactive (32). HNP–1, the most active human defensin, was only approximately 10% as potent as rabbit NP–1 against *C. albicans*. The candida blastospores were protected from defensins by Ca^{2+}, Mg^{2+}, and certain mitochondrial inhibitors, as well as by performing the incubations under anaerobic conditions or at 0°C.

NP–1 and –2 and several other rabbit defensins killed arthroconidia of *Coccidioides immitis* at concentrations of 10 μg/ml or above (33). Fungicidal activity commenced after 4–8 hours of incubation, depended on active fungal metabolism, and failed to occur at 0°C. Rabbit defensins also killed hyphae and germinating spores of *Rhizopus oryzae* and *Aspergillus fumigatus* (34), although they were inactive against dormant, metabolically quiescent spores. Radiolabelled NP–1 was shown to bind to chitin (a component of some fungal cell walls) and to chitin fragments, and these substances protected *A. fumigatus* from the lethal effects of NP–1. The addition of Ca^{2+} but not Mg^{2+} protected the fungi from defensins. The effects of human defensins on these organisms have not been reported.

Spirochetes

Six rabbit defensins were tested against the Nichols strain of *Treponema pallidum*, both in vitro and in vivo. Treponemal neutralization (loss of motility) occurred after incubation with defensins in tissue culture medium that contained 10% normal or heat-inactivated rabbit serum (35), but neutralization was more rapid and extensive in normal rabbit serum. Reduced and alkylated defensins were inactive. C6-deficient serum resembled heat inactivated serum in its ability to support defensin-mediated treponemal neutralization. All six rabbit defensins abrogated or delayed the development of cutaneous lesions, following their intradermal injection into rabbits after an in vitro incubation with *T. pallidum*. The in vivo defensin response was studied in a rabbit model of treponemal orchitis (36). Large amounts of cell-associated and extracellular defensins accumulated locally during the first 24 hr, subsided by day 4, and reappeared between days 10–16, when healing became evident. Differential staining for NP–1 and –2 and NP–5 pinpointed neutrophils as major sources of these defensins, and also suggested a role for these cells in the resolution of early syphilitic lesions.

Mycobacteria

Although the activity of defensins against *M. tuberculosis* has not been described, defensins are active against non-tuberculous mycobacteria, including *M. fortuitum* spp. (R. Lehrer, P. Hiemstra, unpublished) and *M. avium-intracellulare* (37). HNP–1 was effective at concentrations of 5–50

μg/ml against 10/10 strains, including both opaque and transparent colony types. Mycobactericidal activity was unaffected by divalent cations or physiological salt concentrations, and HNP–2 and HNP–3 were about as effective as HNP–1.

Viruses

Many defensins, including rabbit NP–1 and –2 (38), human HNP–1, –2, and –3 (39), rat RatNP–1 (13), and guinea pig GPNP–1 (12) neutralized herpes simplex virus (HSV) in tissue culture media. Viral neutralization was direct, rather than cell-mediated, and was associated with binding of the defensins to the virus (39). The extent of neutralization depended on the concentration of defensin and required intact intramolecular disulfide bonds. Neutralization was also influenced by incubation time, pH, and temperature and could be prevented by adding serum or albumin to the incubation mixtures. Human and rabbit defensins also neutralized vesicular stomatitis and influenza A/WSN virus but lacked significant activity against cytomegalovirus, reovirus, and echovirus.

Antimicrobial Mechanisms

Sawyer and colleagues (40) reported that rabbit defensins NP–1 and NP–2 bound to the surface of *Pseudomonas aeruginosa* PA01 and to purified lipopolysaccharide (LPS) with high affinity. Both defensins permeabilized the bacterial outer membrane (OM), and led to the formation of small bleb-like structures. Outer membrane permeabilization was more effective at pH 6.0–6.5 than at pH 7.0–8.0. The authors explained these effects with reference to the "self-promoted uptake" model previously proposed by Hancock for cationic antibiotics, such as polymyxins and aminoglycosides (41). This model postulates that polycationic molecules displace the divalent cations that normally link adjacent LPS molecules, thereby effecting permeability changes and allowing entry of otherwise excluded molecules such as lysozyme or rifampin. Viljanen and coworkers reported that human defensins increased the permeability of the outer membranes of *P. aeruginosa*, *E. coli*, and *S. typhimurium* to hydrophobic probes such as rifampin and Triton X–100 (42). Although *subinhibitory* defensin concentrations caused little or no permeabilization to rifampin, bacteriostatic or bactericidal defensin concentrations did increase permeability somewhat.

A novel spectrophotometric procedure was used to determine the effects of human defensins on the outer and inner membranes of *E. coli*. Bactericidal concentrations of HNP–1 caused the sequential permeabilization of these membranes, which was followed by a simultaneous cessation of DNA, RNA, and protein synthesis and of respiration (43). Bacterial death

was attributed to the effects of inner membrane permeabilization, which allowed both the loss of intracellular contents and the entry of defensins.

Permeabilization of microbial cell membranes thus appears to be a key consequence of exposure to defensins, and especially with human defensins, it can be inhibited by treatments that either impair the generation of metabolic energy by the target cell or decrease its transmembrane protonmotive force (32, 43). Artificial phospholipid membranes were permeabilized by defensins only when a transmembrane electronmotive force was applied with a polarity that enabled the cationic defensin molecule to insert into the phospholipid bilayer (28). Membrane conductance increased as the second to fourth power of the defensin concentration, suggesting that two to four defensin molecules interacted to form a channel.

We envision the effect of defensins on microbes taking place in three stages. Following the electrostatic adsorption of defensins to sites near the target cell membrane (Stage 1), individual defensin molecules or dimers enter the energized cell membrane under the influence of its electromotive force and disrupt its integrity, probably by forming voltage-regulated pores (Stage 2). Unless these lesions are repaired or removed, irreversible target cell injury occurs, accompanied by the entry of defensins and/or other normally excluded molecules (ions, other peptides and proteins, etc), and by the leakage of essential minerals and metabolites from the target cells (Stage 3). Although future studies may delineate more precisely the subsequent molecular events associated with microbial death during Stage 3, these are very likely to resemble the molecular pathology of a fly hit by a brick.

Defensin Resistance and Pathogenesis

Heffron and associates used transposon mutagenesis to produce *Salmonella typhimurium* strains that showed markedly enhanced susceptibility to defensins in vitro. The mutants, which had disrupted phoP genes, survived poorly after ingestion by murine macrophages and had markedly reduced in vivo virulence (44, 45). The defective phoP gene, a transcriptional activator, was part of a two component regulatory system whose second component, phoQ, was a membrane sensor kinase. Strains that expressed phoQ without phoP were still markedly sensitive to defensins (46). The potential contributions of the phoP/phoQ regulon to Salmonella virulence were recently reviewed (47).

CYTOTOXIC ACTIVITY

Characteristics of the Lytic Reaction

Our initial investigations into cytotoxic activity of defensins were prompted by evidence that human PMNs could lyse mammalian targets via oxygen-

independent mechanisms (48–50). They revealed that purified human defensins killed various human and murine tumor targets in a concentration- and time-dependent fashion (51). Cytotoxicity was first detected after 3 hr. It rose to high levels by 6 hr, there reaching a plateau for some targets while continuing to increase for others until 14 hr. Optimal lysis was achieved with 25–100 μg/ml after 6 hr. However, targets treated with as little as 1 μg/ml of defensin demonstrated up to 50% cytolysis at 14 hr.

Although rabbit defensins NP–1 and NP–2 were reported to damage malignant murine targets without lysing nontransformed murine fibroblasts (52), the human defensins were not tumor-specific and lysed human lymphocytes (51), PMNs (51) and endothelial cells (53) as well as murine thymocytes and spleen cells (51). Human defensins had comparable cytotoxic effects on human lymphohematopoietic and solid tumor targets (A. Lichtenstein, unpublished), and they lysed targets that had been rendered resistant to tumor necrosis factor or NK-cytolytic factor (51). In general, murine cell lines were less sensitive than human lines to the human defensins (51), and nucleated targets were always more sensitive than erythrocytes (54). The only target highly resistant to defensin-mediated cytotoxicity in our hands was a murine cell line, L929 (55).

Remarkable synergistic cytolysis ensued when defensin-containing granule extracts or purified defensins (but not elastase and cathepsin-G) were combined with sublytic concentrations of hydrogen peroxide (54). Synergism occurred with concentrations of defensins and H_2O_2 as low as 5 μg/ml and 10^{-5}M, respectively. The LD_{50} for H_2O_2-mediated lysis in the presence of 5 to 40 μg/ml of defensin was decreased 2.5- to 40-fold, with increasing synergy apparent as H_2O_2 concentrations rose. Addition of sublytic concentrations of H_2O_2 caused increased binding of defensin, presumably reflecting facilitated entry of defensin into the membranes or the intracellular milieu of the targets. Because defensins and H_2O_2 are both secreted by activated neutrophils, such interactions could contribute to leukocyte-mediated cytotoxic effects in vivo.

Mechanism of Injury

At least three sequential interdependent events appeared to be required for target cell lysis. The first two, membrane binding and permeabilization, were extensively characterized (55, 56). The third event may involve DNA injury (57). Although the relative cytolytic and antimicrobial effectiveness of individual human and rabbit defensins were generally correlated, these properties were not simply determined by the peptide's net positive charge, as certain highly cationic defensins were nonlytic and poor microbicides (51, 10). Mammalian target cells were protected from defensins by serum

(55), increasing the extracellular Ca^{2+} (but not Mg^{2+}) concentration, inhibiting target cell microfilament function with cytochalasin-B, disrupting their energy metabolism with a combination of 2-deoxyglucose and sodium azide, or by performing the assay below 27°C (55, 56).

Defensin binding to mammalian target cells occurred with biphasic kinetics, similar to those previously reported with *C. albicans* (30). The initial binding phase occurred within 2 min. The secondary phase began by 10 min and then gradually increased and reached a plateau after 60 min. K562 cells exposed to 20 μg/ml of radiolabeled HNP–1 bound approximately 4.3×10^9 defensin molecules per cell. This initial binding was attributed to relatively weak electrostatic forces between the cationic defensins and negatively charged external surface molecules of the target. The initial binding step was insufficient to cause cytolysis, as its extent was comparable in defensin-resistant (L929) and defensin-sensitive target cells. However, conditions that impaired binding, such as low temperature, presence of serum, or addition of heparin (51) effectively blocked cytolysis.

During the first hour, it became progressively more difficult to dissociate membrane-bound defensin molecules from the target cells by adding serum (55). This time interval correlated with the second (permeabilization) phase of lysis, when defensin-created membrane channels appeared and were stabilized. The developing resistance of target cell–bound defensins to serum-induced dissociation may reflect their insertion into the plasma membrane or internalization by the cell.

Because a 3 to 4 hr delay existed before defensin-treated targets began to release ^{51}Cr-labelled cytoplasmic components (51), we initially believed that defensins did not rapidly create plasmalemmal lesions and therefore that any internalization of defensins was likely to have occurred via endocytosis. However, because defensins created ion channels in model lipid bilayers (28) and permeabilized the membranes of *E. coli* (43) quite rapidly, we reexamined the initial interactions between mammalian cells and defensins with more sensitive assays (56). Within minutes of exposure, defensin-treated targets demonstrated enhanced transmembrane ion flow that was detected by the collapse of their membrane potential, efflux of rubidium, and blunting of the acidification response induced by nigericin. After a lag of 5 to 10 min, the targets became permeable to trypan blue (molecular weight—960 Da). When their transmembrane potential was decreased by increasing the extracellular potassium concentration, defensin-treated target cells were protected from permeabilization, suggesting that the initial defensin-induced pores were voltage-dependent, as also noted in studies with artificial lipid bilayers (28). Protection of the target cells by metabolic inhibitors, azide and 2-deoxyglucose, was also consistent with

the hypothesis that defensin-mediated injury to mammalian cells depended on an energized target cell membrane, much as described for *E. coli* and *C. albicans*.

The early permeabilization phase was also prevented by adding cytochalasin-B. Like the combination of azide and 2-deoxyglucose, this did not affect initial defensin binding, but it prevented subsequent target cell death. The mechanism whereby cytochalasin-B protects target cells from defensin-mediated permeabilization remains uncertain. Possibly, cytochalasin-sensitive cytoskeletal structures or cell motility allow the initial defensin pores or channels to enlarge, promoting either the entry of defensins to critical intracellular sites or the loss of essential target cell macromolecules.

Although the impaired membrane integrity occurring during the first 30 min in defensin-treated targets was initially reversible (56), it was succeeded by a third phase of injury that led to cell death. This final and lethal phase began after 30 to 60 min of incubation and required the continued presence of defensin. It was enhanced by actinomycin-D or cycloheximide, suggesting that de novo protein synthesis provided some protection, perhaps by allowing membrane repair. Increased extracellular Ca^{2+} concentrations also provided protection, perhaps also by promoting membrane repair, as noted with respect to recovery from sublethal transmembrane damage caused by pores produced by complement components C5b–9 (58). The progress of Phase 3 was also inhibited by certain metabolic inhibitors (azide with 2-deoxyglucose), suggesting that cell death did not result simply from leakage of critical metabolites through fixed membrane channels. The requirement for a continued defensin presence may signify that internalization of defensin is required to administer some sort of molecular coup-de-grace, or that defensin-mediated binding is reversible until the lethal step (or stage) is reached.

As had also been noted with *E. coli* (43), protein synthesis was maintained normally in defensin-treated mammalian target cells until the time of their death (55). Defensins damaged target cell DNA, inducing single-strand breaks that were detected in the alkaline unwinding assay, and by activation of poly (ADP-ribose) polymerase, a DNA repair enzyme (57). Apoptosis, the dissolution of DNA into nucleosome-sized fragments, was not detected. If repair of single-strand breaks was inhibited, defensin-induced target cell lysis was enhanced. Such findings suggest that defensin-induced DNA injury may be a causal participant in cytolysis, rather than a nonspecific accompaniment. It is not yet known if the death of defensin-treated targets results from the occurrence of a single, critical, irreversible step or if it reflects overall failure of the complex web of interacting systems

required for cell viability. If there is a specific lethal event, it has not yet been identified.

Relationship to Cellular Cytotoxicity

Although most studies of granulocyte-induced cellular cytotoxicity have emphasized the role of oxidants in this process, it is certain that PMN-mediated target lysis can also occur independently of granulocyte oxidative metabolism. Considering that human (albeit not mouse) PMN contain large amounts of potently cytocidal defensins that can be released externally by various stimuli (59), the participation of defensins in extracellular target cell lysis or injury mediated by human PMN seems likely .

The role of nonoxidative lytic mechanisms appears especially prominent when PMN injure targets by antibody-dependent cell cytotoxicity (ADCC) (48–50, 60–64). Furthermore, certain data strongly suggest that these nonoxidative ADCC mechanisms are mediated through release of cytotoxins from PMN granules. When PMN degranulation was prevented by pharmacological agents (63, 64), ADCC was blocked. Inhibition of lysis and myeloperoxidase release (but not lysozyme release) were correlated, suggesting that release of primary ("azurophil") granule contents was the critical event. It should be noted that defensins are even more abundant than myeloperoxidase in the primary granules of human neutrophils (65). When the cytolytic effects of the various constituents of PMN granules were compared against human leukemia (54), carcinoma (53), and lung fibroblasts (53), defensin-containing fractions proved to be the most cytotoxic. In contrast, fractions containing elastase, cathepsin-G, and other proteases were minimally active, while myeloperoxidase-and lysozyme-containing preparations were inactive. The cytotoxicity of whole human PMN granule extracts against these targets could be almost completely accounted for by their defensins content. Although defensin-containing fractions as well as purified peptide were also cytocidal towards human umbilical vein endothelia, these targets were more sensitive to the protease-containing fractions. This is consistent with the known sensitivity of endothelial cells to elastase and cathepsin G (66, 67). In the PMN ADCC system against human neuroblastoma cells, where lysis is mediated by degranulation (64), the granule proteins responsible for target cell death were shown to be defensins and cathepsin-G (64). Similar inhibition of ADCC was detected when PMNs were depleted of granules by cytoplast formation (68). Overall, these experiments provide strong circumstantial evidence that release of defensins as part of the degranulation process is involved in some in vitro systems of neutrophil cytotoxicity.

For defensins to participate in cellular cytotoxicity, they must be released

in potentially lytic concentrations and then protected from certain naturally occurring inhibitors, including albumin (55) and alpha–2 macroglobulin (69), that are found in many biological fluids. Exposure of approximately 2×10^7 PMN per ml to the soluble secretagogue phorbol myristate acetate (59) would lead to accumulation of approximately 5 to 10 μg/ml of defensin extracellularly, a concentration that could cause significant cytolysis and/or injury to most of the targets we have tested (see above). Much release of defensins appears to take place near the zone of contact between PMN and targets (A. Lichtenstein, unpublished). Such focussed release of cytotoxic defensins at zones of target-effector cell contact could significantly enhance the local concentration of defensin that initially interacts with a target call membrane. Wright & Silverstein (70) showed that the zone of contact between a macrophage and its antibody-opsonized target will exclude serum proteins of 50,000 mol wt or greater. If the contact zone between PMN and antibody-coated targets in ADCC has similar exclusionary properties, then neither albumin nor α–2 macroglobulin would be likely to gain access to the zone where the PMN and target cell membranes are apposed. Thus protected from inactivation by serum, locally secreted defensins could allow the PMN to fulfil an assassin's role while sparing bystander cells.

MOLECULAR BIOLOGY OF DEFENSINS

Structures of cDNAs and Genes

cDNAs for seven myeloid defensins (rabbit NP–1, –2, –3a, –4, and 5; human HNP–1 and –3) and a mouse intestinal defensin (cryptdin) have been cloned and sequenced (22, 71–74). In each case, the mature defensin sequence constituted the carboxy terminus of a prepropeptide, containing a typical amino-terminal 19 residue signal sequence followed by a 40–45 residue propiece. In myeloid defensins, the number of anionic propiece residues (glutamic and aspartic acids) approximately balanced the cationic residues (principally arginine) on the mature defensin peptides (75), possibly affording protection from autocytotoxicity during defensin synthesis (76).

Genes that encoded rabbit defensins NP–1 (also called MCP–1), NP–2 (MCP–2), and NP–3a were mapped and sequenced. The transcribed segments of these genes consisted of three exons each corresponding approximately to the 5′ untranslated region, signal sequence/propiece, and mature defensin region of the mRNA and defensin precursors. The domains 500 bp 5′ to the TATA box of the five known rabbit and human genes were homologous (63% identity) and contained several highly conserved stretches

that may prove to govern developmental and tissue-specific aspects of defensin expression.

Synthesis and Posttranslational Processing

Defensin mRNA was present at high levels in bone marrow, in the human myeloid leukemia cell line HL–60, and in chronic myeloid leukemia (CML) cells (71). Although mature human blood neutrophils contained about 5 μg of defensins per million cells, defensin mRNAs were not detected in Northern blots of these cells (71), indicating that defensin synthesis was restricted to the neutrophil's bone marrow precursors.

Posttranslational processing of defensin was studied in metabolically labelled HL–60 and CML cells (77). Their earliest defensin intermediate contained 75 residues and arose by cotranslational removal of the prodefensin's signal sequence. Considerable quantities of this 75 residue form were secreted into the medium, and the remainder was proteolytically processed over 20 hours via a 56 residue intermediate into the mature 29 and 30 residue defensins. Mature human neutrophils also contained minor amounts (0.25% of total defensins) of incompletely processed 39, 34, and 32 residue defensins, whose significance with respect to the mechanisms of defensin processing remains to be determined (15).

This pattern of synthesis and posttranslational processing was substantially reproduced when the HNP–1 defensin cDNA was transduced into the defensin-less murine myeloid cell line 32D and 32D c13 (L. Liu, E. Valore, and T. Ganz, in preparation). These transgenic cell lines accumulated mature defensin in acidified vacuoles that may correspond to lysosomes or immature granules. In contrast, two nonmyeloid transgenic defensin-producing cell lines, an embryonic NIH 3T3 cell line and the pituitary adenoma cell line AtT–20, failed to process the 75-residue prodefensin, indicating that the requisite processing pathway may be tissue-specific.

Host and Tissue-Specific Expression

Human, rabbit, rat, and guinea pig neutrophils contain large amounts of defensins. Three of the human PMN's four defensins (HNP–1, –2, and –3), comprised between 30% and 50% of the protein in its "azurophil" granules and 5% to 7% of its total cellular protein (65). The other human neutrophil defensin, HNP–4, was approximately 100-fold less abundant (15) and may serve functions other than host defense (78, 21). Defensins constituted $>15\%$ of the total protein in rabbit PMN and were present in a subset of dense cytoplasmic granules (79); in rabbit lung macrophages they constituted approximately 1.5% of the total protein and cosegregated with lysosomal markers (7, 14).

Two of the six rabbit neutrophil defensins, NP–1 and NP–2, were also present in alveolar macrophages, where they were first described as macrophage cationic peptides, MCP–1 and MCP–2 (14). Rabbit alveolar macrophages incorporated ^{35}S-cysteine into these nascent defensins, confirming that they were synthesized (80), rather than accumulated via uptake from other sources. As neither monocytes (macrophage precursors) nor peritoneal macrophages contained defensins, synthesis of macrophage defensin was evidently tissue-specific (7, 73). Messenger RNA for MCP–1 and MCP–2 was abundant in alveolar macrophages but undectable in Northern blots of monocytes or peritoneal macrophages (73), indicating that synthesis was under pretranslational regulation. Although neonatal rabbit alveolar macrophages lacked defensins at birth, they attained nearly adult levels during the first few weeks of postnatal life (80). Immune stimulation of rabbits by intravenous injection of complete Freund adjuvant tripled the cellular content of defensins in alveolar macrophages (81). The regulatory elements that control such developmental, immune, and tissue-specific regulation of defensin gene expression in the rabbit are under study.

Despite a careful search, defensins were not found in mouse or horse neutrophils (82, 83). The former finding, noted in 9/9 mouse strains, was especially surprising since mRNA encoding a typical defensin precursor ("cryptdin") had been cloned from murine small intestine (22, 74), and several mature (i.e. processed) mouse defensins have been purified from this source (23). Two cell populations contained cryptdin mRNA by in situ hybridization: epithelial (Paneth) cells, found at the base of intestinal crypts, and macrophages, found in the lamina propria (74). As in rabbit alveolar macrophages, expression of defensin by small intestinal Paneth cells was developmentally regulated. The intestine of newborn mice had very low levels of cryptdin mRNA, and adult levels were reached by 6 weeks of life (74). Cryptdin mRNA levels were reportedly normal in germ-free and nude mice, suggesting that its expression did not depend on T cell signals or acquisition of an intestinal flora. Although rabbit Paneth cells were recently shown to contain a defensin (NP–3a) (84), whether any defensins are present in human Paneth cells is not yet known.

Evolution

The defensin family includes many peptide species whose expression may vary within the tissues of individuals or their populations. The existence of multiple defensin genes in individual humans or rabbits epitomizes the remarkable diversity of this family among different species. Figure 2 arranges the sequences of nonallelic defensins (defensin forms that do not genetically segregate within populations) in a homology tree, or "dendro-

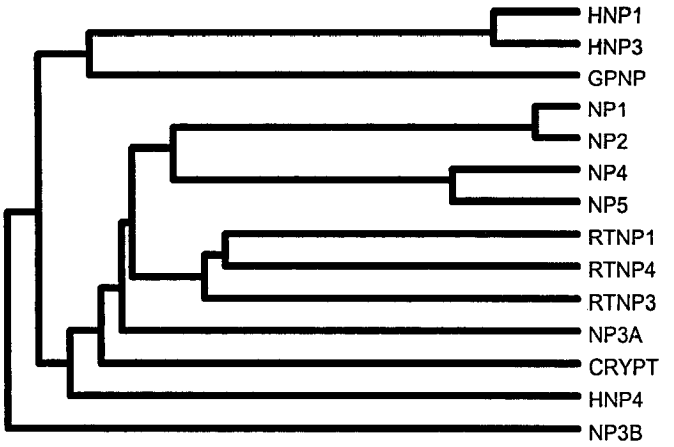

Figure 2 Dendrogram of the known defensin peptides. Length of the horizontal lines connecting two peptides indicates their calculated evolutionary distance. The diagram was generated by the Clustal program (PC-Gene, INteligenetics, Inc.). HNP, human; GPNP, guinea pig; NP, rabbit; RTNP, rat; CRYPT (cryptdin), mouse; TAP (tracheal antimicrobial peptide), bovine.

gram." The pairing of highly homologous peptides in humans, rabbits, and rats suggests that gene duplication has occurred repeatedly during evolution. More direct evidence for gene duplication exists, because the highly homologous rabbit genes NP–1 and NP–2 are located within 13 kb of each other (73). In addition, the two recently sequenced nonallelic human genes, HNP–1 and HNP–3, differ from each other by only a few nucleotide substitutions (T. Ganz and R. Linzmeier, in preparation), and they share the chromosomal locus 8p23 (85). Defensin gene mutations have also resulted in genetically segregating, allelic genes among rats (86). About 10% of human neutrophil samples lack HNP–3 (T. Ganz, unpublished), a remarkably common polymorphism whose biosynthetic mechanism and clinical consequences, if any, remain to be determined. It is not yet known whether the two guinea pig defensins arose from duplicated or allelic genes (13).

Posttranslational mechanisms may also contribute to defensin diversity. For example, although most human neutrophils contain large amounts of HNP–1, –2, and –3, only the genes and cDNAs for HNP–1 and HNP–3 have been identified. It is possible, therefore, that mature HNP–2 arises via proteolytic removal of the amino terminal alanine of HNP–1 or the amino-terminal aspartic acid of HNP–3, or both. Ascertaining how the

diversity of defensin influences host-pathogen interactions remains an important task for the future.

MECHANISMS THAT LIMIT DAMAGE TO HOST HOST

Large numbers of neutrophils infiltrate tissues during acute inflammation. Under such circumstances, the release of potentially cytotoxic defensins could damage surrounding host cells. Several mechanisms may limit the extracellular accumulation of defensins and mitigate host injury. Based on the number of defensin-containing granules in the human neutrophil (about 1000), the volume of an azurophil granule (1.1×10^{-14} ml), and the cell's content of defensins (5×10^{-9} mg/cell), we estimated that the average concentration of defensins in granules approximates 50 mg/ml (87). Microbes ingested by neutrophils and contained in phagosomal vacuoles are exposed to the contents of granules that fuse to phagosomes. Since electron micrographs usually show the phagosomal membrane tightly apposed to ingested microbes, defensins and other granule contents are likely to be present at high concentrations within phagolysosomes.

Although neutrophils release many other granule components after stimulation by soluble or particulate secretagogues, $<10\%$ of the cell's defensin content enters the medium under such conditions (59). Any defensins released from neutrophils in vivo would not only be substantially diluted by extracellular fluid, they would also be exposed to plasma-derived binding proteins that could potentially neutralize their cytotoxic activity (51). In vitro, human defensins bound specifically to an activated form of the plasma protein α2-macroglobulin whose formation was elicited when plasma was treated with primary amines (69) or proteases, including neutrophil elastase (A. Panyutich, T. Ganz, in preparation). Because neutrophil-derived and other proteases are released at inflammatory sites, this observation may have an in vivo counterpart. Because α2-M is taken up by macrophages by means of a receptor-mediated process (88), the uptake of α2-M-bound defensins by macrophages could equip them with defensins for subsequent use against microorganisms or enable them to concentrate and degrade defensins within the lysosomes.

OTHER PROPERTIES OF DEFENSINS

Monocyte Chemotaxis

Human neutrophil defensin HNP–1 was chemotactic for human monocytes in vitro and exerted peak activity at 5×10^{-9} M. HNP–2 was also chemotactic, but HNP–3 was inactive. It was suggested that release of

defensins from neutrophils during infection or inflammation might attract monocytes to the involved sites (89). The defective in vivo monocyte "skin window" response noted in a patient with "specific granule deficiency" (90), a rare disorder associated with a severe deficiency of defensins (91) and other granule components, was consistent with this possibility.

Inhibition of ACTH-Binding

Rabbit NP–3a bound specifically and reversibly to the ACTH receptor of rat adrenal cells in vitro and inhibited ACTH-stimulated steroidogenesis (21, 78, 92, 93). This property has been called "corticostasis" by Solomon and his colleagues, who have also sought to rename the defensins 'corticostatins." Although NP–3a concentrations >2 μg/ml were needed to completely block steroidogenesis, approximately 25 nM caused half maximal inhibition. Of the human neutrophil defensins, only HNP–4 exerts significant corticostatic activity, and it is less potent than NP–3a in this regard (21). The corticostatic mechanisms of defensins were recently reviewed (94). The functional significance of corticostasis remains to be demonstrated. Neither the benefit nor the appropriateness of renaming defensins is immediately apparent.

Inhibition of Protein Kinase C

Human neutrophil defensins are exceptionally potent inhibitors of purified protein kinase C (PKC), acting noncompetitively with respect to phospholipid, Ca^{2+}, ATP, and the substrate, histone H1 (95). Although defensins block activation of PKC by TPA (12–0-tetradecanoyl-phorbol–13-acetate) they do not inhibit its binding to PKC. Unlike most other PKC-inhibitory peptides, defensins fail to inhibit myosin light chain kinase, a calmodulin/Ca^{2+}–dependent enzyme. Neither the precise mechanism of defensin-mediated inhibition of PKC nor its biological significance are currently known.

Miscellaneous Effects

The ability of rabbit alveolar macrophages to ingest bacteria and fungi under serum-free conditions was greatly enhanced by rabbit defensins NP–1 and NP–2 (96). By serving as opsonins, these defensins could enhance the ability of alveolar macrophages to ingest organisms that enter the lung's air spaces. Daily injections of rabbit defensins, 125 μg/kg body weight, were found to accelerate wound healing in rats (97), suggesting that defensins could contribute to subsequent tissue repair at sites of inflammation. Guinea pig defensins have been reported to release histamine from rat mast cells in vitro (11).

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IMMUNITY TO INTRACELLULAR BACTERIA

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Abstract

Intracellular bacteria are endowed with the capacity to survive and replicate inside mononuclear phagocytes (MP) and, sometimes, within certain other host cells. MP are potent effector cells that are able to engulf and kill many bacterial invaders. Therefore, intracellular bacteria had to exploit potent evasion mechanisms that allow their survival in this hostile environment. At the early phase, natural killer cells activate antibacterial defense mechanisms. During intracellular persistence, microbial proteins are processed and presented, thus initiating T cell activation. By secreting interleukins, CD4 α/β TH1 cells activate MP, converting them from a habitat to a potent effector cell; TH2-dependent activities seem to be of minor importance. Cytolytic CD8 T cells represent a further element of protection. In the case of *Listeria monocytogenes*, the gene products responsible for virulence and for the introduction of antigens into the MHC class I pathway are being characterized. Increasing evidence points to a role of γ/δ T lymphocytes in antibacterial immunity, although their precise function remains to be elucidated. Protection in the host is a local event focussed on granulomatous lesions. MP accumulate at the site of microbial growth and become activated through the CD4 T cell–interleukin–MP axis. Lysis of uncapacitated MP and other host cells by CD8 T cells allows release and subsequent uptake by more efficient phagocytes, thus contributing to host protection. At the same time, lysis of host cells promotes microbial dissemination and causes tissue injury, which represent pathogenic aspects

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of the same mechanism. Research on the immune response against intracellular bacteria not only helps us to better understand how the immune system deals with "viable antigens" in constant transmutation, it also forms the basis for the rational design of control measures for major health problems.

INTRODUCTION

The crucial feature of pathogenic bacteria is their capacity to stably infect their host. Eventually, though not necessarily, infection may have the detrimental consequences for the host which are then commonly termed "infectious disease." To fulfill their goal, bacterial pathogens have exploited a variety of niches in the host which allow their survival in face of a potent immune response. One successful strategy is the use of an intracellular habitat which effectively protects them from humoral defense mechanisms. Although practically any cell can be abused as habitat, mononuclear phagocytes (MP) are frequently preferred. Because they are highly phagocytic and long-lived, they fulfill some requirements for a microbial niche. On the other hand, MP are potent effectors of antimicrobial defense, and thus intracellular bacteria have had to develop means that allow their intracellular survival in a hostile environment. The exploitation of intracellular survival mechanisms inside MP is the first distinguishing marker of an intracellular bacterial infection. Living within MP, pathogens avoid antibody responses while processing and presentation of their proteins by MP promote T cell stimulation. While it is well appreciated that T lymphocytes are instrumental in acquired resistance, it is becoming increasingly clear that they also participate in pathogenesis. Thus, to a large extent, pathogenesis of these infections is not a direct corollary of microbial virulence factors but rather of the host response. The crucial role of T lymphocytes represents the second characteristic marker of intracellular bacterial infections. Microbes growing in the extracellular space cause accumulation of polymorphonuclear granulocytes (PNG), which results in purulent reactions. In contrast, tissue reactions to intracellular bacteria are typically granulomatous, this representing the third distinctive marker of this type of infection. Although the intracellular bacteria are successfully contained inside these granulomas, sterile eradication often fails to occur. Hence, the disease becomes chronic, this being the fourth marker of intracellular bacterial infections.

Only a few bacterial pathogens fulfil these four criteria, and thus, depending on how stringently standards are set, the list of intracellular bacteria will be of different length (1; and Table 1). The simple capacity to enter host cells is an obligatory, yet insufficient criterion since many

Table 1 Infections caused by intracellular bacteria

Pathogen	Disease	Port of entry	Contribution to protection of cellular/humoral immunity	Evasion mechanism
Mycobacterium tuberculosis/M. bovis	Tuberculosis	lung	+++/−	Inhibition of phagosome-lysosome fusion, interference with ROI, resistance against lysosomal enzymes
Mycobacterium leprae	Leprosy	oral mucosa	+++/−	Evasion into cytoplasm (?), interference with ROI
Listeria monocytogenes	Listeriosis	gut	+++/−	Evasion into the cytoplasm
Salmonella typhi/S. paratyphi	Typhoid	gut	++/+	Resistance against lysosomal enzymes, inhibition of phagosome-lysosome fusion interference with ROI
Legionella pneumophila	Legionnaire's Disease	lung	+/++	Inhibition of phagosome-lysosome fusion, interference with ROI

extracellular bacteria such as *Shigella* sp. enter the host by transient passage through epithelial cell layers. It is unequivocal that the etiologic agents of tuberculosis, *Mycobacterium tuberculosis* and *M. bovis*, best fulfil all criteria. This is similarly true for *M. leprae* which, however, complicates the situation by markedly interfering with the T cell response. This matter has been discussed elsewhere and is not considered here (2, 3). Experimental listeriosis of mice, which is caused by *Listeria monocytogenes*, fulfils almost all the criteria but one, i.e. the disease has an acute course. Other diseases such as typhoid caused by *Salmonella typhi*/*S.typhimurium* or Legionnaire's disease caused by *Legionella pneumophila* are only partially intracellular bacterial infections because much of the host response is influenced by antibodies. No wonder that, besides general commonalities, intracellular bacterial infections show remarkable individual variations. In this review, therefore, emphasis is laid upon two infections of quite different character: First, human tuberculosis is considered because it not only paradigmatically represents an intracellular bacterial infection but also because it causes major health problems. No attention is paid to specific differences between *M. tuberculosis* and *M. bovis*. Second, emphasis is given to experimental listeriosis in mice because this represents the best studied model of intracellular bacterial infections.

THE INFECTIOUS AGENTS AND THEIR DISEASES

M. tuberculosis/*M. bovis* are acid-fast intracellular bacteria with a replication time of $\geqslant$ 12 hr. They are obligate aerobes that prefer tissue sites with high pO values, the lung in particular. *M. tuberculosis*/*M. bovis* as well as other mycobacteria contain abundant lipids and waxes, which are responsible not only for their hydrophobic character, but also for their strong adjuvanticity and intracellular persistence. *M. tuberculosis* and to a small extent *M. bovis* cause tuberculosis, which is of paramount medical importance globally (4). This disease is characterized by a long incubation time, dormant infection, and chronic course. Globally 60 million people are estimated to suffer from tuberculosis, and every year about 10 million new cases arise. Annually, more than 3 million people die of this disease, giving *M. tuberculosis* the highest morbidity rate among all infectious agents. At the same time it has been estimated that 1/3 to 1/2 of the whole world population (approximately 2 billion people) are infected with *M. tuberculosis*. Most of these infected individuals harbor dormant microbes within small granulomas. Persistence of *M. tuberculosis* at these secluded sites does not remain unrecognized by the immune system; rather, it is controlled by T cells. Hence, in the vast majority (>90%) of infected people a long-lasting but labile balance between host immune response and

persistent mycobacteria develop. As long as this balance is equilibrated, the infected individual remains healthy. In a minority of infected individuals, weakening of the immune system at a later time tips the balance in favor of the microbe, and disease develops after reactivation of foci harbouring *M. tuberculosis* organisms. Infection generally occurs via the aerogenic route, and the lung is the prime site of infection and disease. Nevertheless, any other tissue site can be infected after reactivation of dormant foci and hematogenic/lymphogenic dissemination.

L. monocytogenes is a grampositive, non-spore-forming, aerobic to microaerophilic rod (5). Recent molecular biological analyses have revealed several virulence factors of *L. monocytogenes* which are instrumental in invasion and intracellular replication (6).Listeriosis is primarily a disease of sheep and cattle and is seldom seen in humans (5). Prenatal and neonatal infections that generally cause disseminated abscess and granuloma formation are of some medical importance, and certain immunocompromised patients show increased incidences of listeriosis. Scattered food-borne outbreaks of listeriosis which also affect apparently healthy adults have been noted more recently. *L. monocytogenes* is of particular interest because experimental listeriosis of mice is a widely studied model for intracellular bacterial infections. It is an acute infection that is readily overcome once T cells have been activated (1). Accordingly, listeriosis differs substantially from tuberculosis both in humans and mice. Even allowing for such differences, however, this system has proven highly useful for analyzing cell-mediated antibacterial immunity.

INTRACELLULAR KILLING AND SURVIVAL

Professional Phagocytes as Major Effectors of Antibacterial Defense

Professional phagocytes comprising polymorphonuclear granulocytes and mononuclear phagocytes are the major effectors of antibacterial defense. Although the role of polymorphonuclear granulocytes has often been neglected in considering immune defense against intracellular bacteria, PNG play a special part in acute intracellular infection (1, 7–10). Their limited life-span of approximately 1 day, however, makes them unsuitable as an intracellular habitat. Rather, their highly aggressive potential makes them potent killer cells for intracellular bacteria such as *L. monocytogenes*. Furthermore, the highly secretory PNG also participate in tissue destruction and granuloma liquefaction (7, 11).

The role of mononuclear phagocytes in intracellular bacterial infection is twofold because they represent both an essential habitat and the major effectors of defense. MP are extremely heterogeneous with respect to their

antibacterial capacity, depending on tissue localization and activation by exogenous stimuli. Their long life-span of months and their limited antibacterial capacity make resident tissue MP ideal candidates for colonization by highly resistant intracellular bacteria such as *M. tuberculosis*/*M. bovis*.

The major effector functions of MP include: generation of reactive oxygen intermediates (ROI); production of reactive nitrogen intermediates (RNI); limitation of intracellular iron availability; phagosome acidification plus phagosome-lysosome fusion; and production of defensins. Most of these mechanisms are only induced by appropriate activation which, in the case of murine MP, is optimally achieved by IFN-γ stimulation, although other interleukins may also participate (12–14). The situation in the human system is more complex, and it is probable that optimum activation of tuberculostatic capacities, perhaps the most demanding capacity of MP to achieve, is only accomplished by costimulation with different factors (15–18).

Reactive oxygen intermediates are toxic for microbial organisms including intracellular bacteria. The contribution of ROI in combatting tuberculosis, however, remains controversial (17, 19, 20). For example, addition of a variety of ROI scavengers fails to interfere with intracellular growth inhibition of *M. bovis* by IFN-γ-stimulated murine MP (20). More recently, convincing evidence has been published that RNI are crucially involved in tuberculostatic activities of murine MP (21–24). RNI are produced not only by professional phagocytes, but also by a variety of other cells including hepatocytes, a target cell of *L. monocytogenes* (25). It is, therefore, possible that RNI participate in self protection of certain nonprofessional phagocytes against intracellular bacteria. The role of RNI in human MP remains controversial, although participation of RNI in mycobacterial growth inhibition by human MP has been claimed (26).

Iron is crucially required for intracellular bacterial survival. Iron-saturated transferrin is transported into the cell via transferrin receptors and stored intracellularly bound to ferritin. Limitations in the available intracellular iron by downregulation of transferrin receptors and/or decrease of intracellular ferritin levels limits intracellular survival of *L. monocytogenes* and *L. pneumophila* (27, 28). On the other hand, MP require iron for activation of antibacterial mechanisms including iron-dependent ROI and RNI generation. Thus, competition for iron represents a crucial factor in the decision as to whether a MP serves as habitat or effector.

Lysosomes contain numerous polypeptides with antimicrobial activities. While some of these have direct microbicidal activities, the majority of lysosomal polypeptides are involved in degradation of microbes killed by other mechanisms. During phagocytosis, the endosomal pI is increased to

basic levels for a brief period that provides optimum conditions for defensins. Soon thereafter, the pI decreases to acidic levels thus supporting optimum activity of numerous acidic lysosomal enzymes. Defensins are basic polypeptides comprising 29–34 amino acids which are abundant in PNG and present in some, though not all, MP (19). Purified defensins possess potent microbicidal activity and may play a role in defense against certain intracellular bacteria such as *S. typhimurium* and *L. monocytogenes*. The phoP$^-$ transposon mutants of *S. typhimurium*, which are avirulent for mice and fail to survive in murine MP, were found to be highly sensitive to defensins (29).

Intracellular Survival Strategies

The capacity to replicate inside host cells denotes the distinctive pathogenic feature of intracellular bacteria which forms the basis of all subsequent protective and pathologic events. Different means have been exploited to achieve this goal including: invasion of nonprofessional phagocytes; evasion into the cytoplasmic compartment; interference with ROI; inhibition of phagosome-lysosome fusion and neutralization of phagosomal pI; resistance to lysosomal enzymes; and induction of heat shock proteins.

Bacterial entry into MP proceeds by normal phagocytosis, i.e. via broad reactive receptors for mannose and/or fucose, via Fc receptors (FcR) after immunoglobulin binding or via complement receptors (CR) after deposition of C3b or other complement breakdown products (30). Uptake via FcR induces ROI production and hence may contribute to antimicrobial resistance. In contrast, uptake via CR causes endocytosis in the absence of ROI generation and thus facilitates bacterial survival. Cell-invasive bacteria express specific molecules called invasins which mediate adherence to and subsequent endocytosis by nonprofessional phagocytes (31). Invasive bacteria frequently abuse physiological receptors such as the integrins and the epidermal growth factor receptor (32, 31).

The cellular response to various exogenous assaults comprises the increased synthesis of a group of polypeptides which have been termed heat shock proteins (hsp) because heat has been most often used as insultive signal (33, 34). Many of the assaults known to induce hsp synthesis in vitro also occur inside MP; these include pI and pO changes, Fe deficiency, and ROI production, suggesting that intracellular bacteria produce elevated hsp levels inside MP. Increased hsp synthesis renders the cells more resistant to subsequent, otherwise lethal, insults. These findings indicate that intracellular bacteria increase their hsp synthesis to protect themselves against intracellular killing. Consistent with this notion, *S. typhimurium* mutants with deficient hsp gene expression are more suseptible to intracellular killing, and hsp levels are increased in *S. typhimurium* residing in

MP but not in nonprofessional phagocytes (35, 36). Loss of in vivo virulence of *S. typhimurium* mutants has also been associated with a defective hsp gene (37). Although direct evidence for coordinate expression of hsp and other virulence factors is missing, circumstantial evidence suggests some degree of cross-talk. Thus, heat shock induces ROI detoxifying enzymes in *S. typhimurium* and increases listeriolysin (Hly) synthesis in *L. monocytogenes* (38–40). The direct and rapid response of hsp gene expression to insultive signals would not only make this system an ideal sensor for coordinate virulence gene expression; it also provides an effective system for high level expression of foreign antigens by recombinant viable vaccines (41–43).

L. monocytogenes and *M. tuberculosis/M. bovis* have chosen different survival strategies that are probably directly related to the longevity of disease they cause. *L. monocytogenes* which causes acute infections is relatively sensitive to killing by phagocytes and can only survive inside nonprofessional phagocytes and those few MP which are insufficiently qualified. In contrast, *M. tuberculosis/M. bovis* organisms adopted MP as their major habitat and only rarely inhabit nonprofessional phagocytes.

L. monocytogenes: Active invasion of nonprofessional phagocytes is mediated by an invasin called internalin and an extracellular protein, p60 (44, 45). This invasion mechanism is important for transcytosis of *L. monocytogenes* through intestinal epithelial cells, whereas its relevance to hepatocyte invasion remains unclear. In the liver, *L. monocytogenes* is cleared from the blood by Kupffer cells which in their nonactivated stage are of low antibacterial capacity (46, 47) and at least some bacteria can leave the phagosome and enter the cytoplasm. Although Hly seems to be of major importance, other virulence factors including phospholipase, lecithinase, and metalloprotease apparently also contribute to intracellular survival (6, 48). Subsequently, *L. monocytogenes* moves to the cell membrane from where it spreads to hepatocytes without exposure to the extracellular milieu and independent of invasins (49). Following IFN-γ stimulation of MP, *L. monocytogenes* is no longer able to escape into the cytoplasm (50). It remains within the phagosome where it is readily killed. In-vitro survival of Hly^- mutants in murine MP is markedly reduced, whereas in murine hepatocytes they grow in an almost unrestricted fashion (G. Szalay, S. H. E. Kaufmann, unpublished). Thus, the endosomal compartment of hepatocytes is far less hostile than that of MP, thus providing an amiable niche for bacterial survival.

M. tuberculosis/M. bovis: The major habitat of *M. tuberculosis/M. bovis* is inside granulomatous mononuclear phagocytes. Invasins are not known to exist in these bacteria and may not be required since host entry commences with phagocytosis by the alveolar MP. Evasion into the cytoplasm

does not occur generally, although it has been reported in certain situations (51). *M. tuberculosis/M. bovis* is highly resistant to the effector mechanisms which operate in activated MP. Despite their great medical relevance, little is known about the virulence factors allowing intracellular survival of *M. tuberculosis/M. bovis*. Perhaps this has something to do with the fact that those nonproteinacious entities which are of central importance to virulence do not readily lend themselves to genetic analyses.

M. tuberculosis/M. bovis supports its own phagocytosis by MP via two mechanisms. First, these mycobacteria secrete abundant quantities of fibronectin binding molecules of 30–32 kDa and 57–60 kDa which promote phagocytosis by fibronectin receptors (52, 53). Second, *M. tuberculosis* induces C3 activation followed by deposition of C3 products and uptake via CR (54). This entry is relatively safe because it by-passes reactive oxygen intermediates induction. *M. tuberculosis/M. bovis* also produces ROI detoxifying enzymes and inhibits phagosome-lysosome fusion. NH_4^+ is involved in the neutralization of the acidic endosomal pI, in blocking of ROI production and in inhibition of lysosome movements and fusion with phagosomes (55, 56). Glycolipids also participate in survival of the tubercle bacilli. Lipoarabinomannan interferes with MP priming/activation and sulfolipids inhibit phagosome-lysosome fusion and interfere with ROI production (57–60). Finally, by virtue of its lipoid rich cell wall, by reducing its metabolic activities and further delaying replication, *M. tuberculosis/M. bovis* seems to be adequately equipped for intraendosomal survival.

THE IMMUNE RESPONSE

The Role of Interleukins

IFN-γ IFN-γ plays a central role in acquired resistance of mice against intracellular bacteria. It is produced by all mediators of resistance, namely CD4 T cells, CD8 T cells, γ/δ T cells and natural killer cells (NK) (61–69). Factors produced by *L. monocytogenes*-reactive CD4 T cell clones in vitro are capable of adoptively transferring resistance to the disease with the relevant molecule being tentatively identified as IFN-γ (65, 70). Administration of recombinant IFN-γ protects mice against *L. monocytogenes* and lethal *M. tuberculosis* infection while neutralization with anti-IFN-γ antibodies markedly exacerbates the diseases (71–73). IFN-γ has been identified in sera of *L. monocytogenes*-infected mice and in pleural fluids of patients with tuberculous pleuritis (63, 74). Local application of IFN-γ into lepromatous skin lesions causes an influx of monocytes and T cells to and promotes granuloma formation at the site of application (75). That these IFN-γ-induced events are protective is indicated by reduced numbers of viable *M. leprae* organisms at the site of injection.

Although attempts to stimulate listericidal activities in murine inflammatory MP with IFN-γ in vitro have frequently failed, stimulation of resident peritoneal MP or bone marrow-derived MP with IFN-γ induces antilisterial activity (76, 77). IFN-γ is also a potent stimulator of tuberculostasis in murine MP, although certain *M. tuberculosis* strains are resistant (12, 21). Attempts to stimulate tuberculostasis in human MP with IFN-γ in vitro have frequently failed (16–18). Evidence has been presented for an involvement in tuberculostasis of 1,25-dihydroxyvitamin D3, the biologically active form of vitamin D3 (16, 18). This molecule is synthesized in the skin under UV light or taken up with diet (78) and then hydroxylated at C25 to become the circulating metabolite 25-hydroxyvitamin D3 (78). 1α-hydroxylase activity has been detected in activated MP, and increased levels of 1,25-dihydroxyvitamin D3 were found in pleural fluids of patients with tuberculous pleuritis (79–81). It is likely that IFN-γ-stimulated MP convert 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 in situ which then acts as costimulator of tuberculostasis. An additional participation of TNF is likely (15).

TNF TNF is secreted by human MP after stimulation with mycobacteria, and proteins as well as lipoarabinomannan have been implicated as stimulators (82–85). Differences in TNF secretion by structurally different lipoarabinomannan have been associated with differences in virulence (85). High TNF levels have also been identified in pleural fluids of patients with tuberculous pleuritis and in sera of mice suffering from lethal listeriosis (74, 86).

Treatment of mice with anti-TNF antibodies or with soluble recombinant TNF receptors, dramatically exacerbates *L. monocytogenes* infection (87, 86). Conversely, TNF application causes resistance to an otherwise lethal infection with *L. monocytogenes* (88). Although TNF alone is incapable of activating tuberculostatic capacities in murine MP, it shows marked synergy with IFN-γ (13). Furthermore, IFN-γ–induced tuberculostasis is blocked by anti-TNF antibodies (I. E. A. Flesch, S. H. E. Kaufmann, unpublished). These findings suggest that IFN-γ induced tuberculostasis depends on two distinct intracellular signalling pathways, one being TNF mediated. Administration of anti-TNF-α antibodies was found to decrease resistance of mice against lethal *M. tuberculosis* infection but was without effect on secondary infection of already immune mice (71). An involvement of TNF in the formation of tuberculoid granulomas can be inferred from studies by Kindler et al who observed local TNF synthesis in *M. bovis*-induced granulomas of mice (89). Application of anti-TNF antibodies inhibited granuloma formation, and, as a corollary, abundant mycobacteria were detected in the livers of the treated animals.

TNF is involved in septic shock which is often lethal (90). Because intracellular bacteria are focussed to distinct foci, systemic shock reactions are generally avoided during these infections. However, steady TNF production during chronic tuberculosis may be responsible for tuberculous cachexia. Furthermore, by affecting important organ functions TNF can contribute to pathogenesis of intracellular bacterial infections.

IL–1 During *L. monocytogenes* and *M. tuberculosis*/*M. bovis* infection IL–1 levels are increased (82, 91). Application of exogenous IL–1 increases resistance of mice to *L. monocytogenes*, and treatment of mice with anti-IL–1 receptor antibodies exacerbates listeriosis (92–93). Its major function in antilisterial resistance seem to be associated with phagocyte influx at the early stage of defense (93).

IL–4 IL–4 is capable of activating listeriocidal and tuberculostatic functions in murine MP (13, 94). Mononuclear phagocytes stimulated with IL–4 before infection with mycobacteria fail to inhibit intracellular growth of *M. bovis*. However, when given after infection, IL–4 is capable of reducing mycobacterial growth to a significant degree. Recently, it was found that treatment of mice with anti-IL–4 antibodies markedly increases resistance to listeriosis and inhibits development of lesions in livers; control by IL–4 of inflammatory phagocytes has been proposed (95). IL–4 induces monocyte influx in vivo and formation of multinucleated giant cells in vitro (96, 97) and in this way may contribute to formation and stabilization of tuberculous granulomas.

OTHER INTERLEUKINS Other interleukins including IFN-α, IL–2, IL–6, IL–8, and CSF participate in the immune response to intracellular bacteria (98, 99). IL–6 is produced during *L. monocytogenes* and *M. bovis* infection (100, 101). Also, IL–6 production by blood MP from *M. tuberculosis*-infected subjects was found to be increased (83). IL–6 induces antimycobacterial and listericidal activities in murine MP in a way similar to IL–4 (14). IL–8 is produced by MP and other cells, and a recent study showed IL–8 secretion by human MP lines following phagocytosis of *M. tuberculosis* (102). Although the role of IL–8 in intracellular bacterial infections needs to be further elucidated, at the time being it appears that it is involved in the development of lesions. At early stages, it may temporarily attract PNG to the lesions; later, by attracting T lymphocytes, it may contribute to granuloma formation (103, 104). During murine listeriosis the colony-stimulating factors, G-CSF, M-CSF, and GM-CSF are produced which may play a role in the attraction and activation of phagocytes early after infection (105–107).

Analyses with Scid Mutants: The Role of Natural Killer Cells

Studies using T cell– and B cell–deficient scid mice have been instrumental to analyzing the role of T cell–independent mechanisms in resistance against *L. monocytogenes* (62). Earlier studies employing T cell deficient nu/nu mice had already indicated the existence of potent effector mechanisms in the absence of T cells (108). The nu/nu and scid mice control listeriosis quite effectively in the beginning. However, granulomatous lesions are not formed and over time, these mice will succumb to listeriosis. It was found that T cell functions are partially compensated for by NK cells in scid mice. Upon infection with *L. monocytogenes*, MP produce high levels of TNF and IL–1 (92, 109). TNF, probably together with *L. monocytogenes* components, induces IFN-γ production in NK cells, which then stimulates increased listericidal activities in MP (110). In the long run, however, NK cells do not produce all the factors required for granuloma formation and sterile elimination of *L. monocytogenes*. NK cells are also stimulated in normal mice after *L. monocytogenes* infection, and the highest IFN-γ levels are observed during the first few days of infection (63, 111, 112). It is, likely, therefore, that similar mechanisms are operative in normal mice until *L. monocytogenes*-specific T cells enter the stage at day 4 to 5 (113, 114).

α/β T Lymphocytes

The T cell system can be separated into three stable subsets according to their use of T cell receptors (TCR) and accessory molecules which interact with the MHC gene products on target cells (115). These are CD4 α/β T cells which recognize antigenic peptides in the context of MHC class II; CD8 α/β T cells which recognize antigenic peptides in the context of MHC class I; and γ/δ T cells which are mostly double-negative, i.e. lack the CD4 and the CD8 molecule. According to their interleukin secretion pattern, CD4 T cells can be further separated into TH1 cells which produce IL–2/IFN-γ and TH2 cells which secrete IL–4/IL–5 (116). The central role of T lymphocytes in acquisition of resistance against intracellular bacteria was established before separation into α/β T cells and γ/δ T cells was possible. Yet, it is likely that most of the effects observed can be ascribed to α/β T cells which make up more than 90% of all T cells in peripheral blood and lymphoid organs of mouse and human. However, the central role of α/β T cells in protection against murine listeriosis has been questioned more recently (117).

CD4 T CELLS The central role of class II–restricted CD4 T lymphocytes in immunity to many intracellular bacteria is without doubt. In fact, one of the first reports on class II–restricted T cell functions was based on

experiments with *L. monocytogenes*-infected mice (118). Upon restimulation with antigens of the homologous infectious agent, CD4 T cells from *L. monocytogenes*- or *M. tuberculosis/M. bovis*-infected mice produce IL–2 and IFN-γ and hence are of the TH1 type (65, 66, 99, 119–121). Antigen-specific CD4 TH1 cells have also been consistently isolated from patients suffering from intracellular bacterial infections such as tuberculosis or leprosy (69, 122–125). Bacteria-reactive TH1 cells also express cytolytic activities both in the murine and the human system (69, 126–128). Using more sensitive detection assays, the TH2 cell–derived interleukin IL–4 has been detected in mice immunized with mycobacteria and listeriae (87, 129). This finding raises the question about a TH1/TH2 cell interplay in the regulation of intracellular bacterial infections as known from experimental infection with the intracellular protozoon, *Leishmania major* (130). In this system, IFN-γ neutralization promotes TH2 cell development and increases susceptibility to infection, whereas IL–4 neutralization supports TH1 cell development and elevates resistance. In contrast, no evidence for disturbances in the TH1/TH2 cell balance was observed in *L. monocytogenes*-infected mice after treatment with anti-IL–4 antibodies, although this treatment markedly increased antilisterial resistance (95). For the time being, negative control of TH1 cells seems to be of minor relevance to immunity against listeriosis and tuberculosis. In these infections, IFN-γ is produced not only by TH1 cells but also by NK cells, γ/δ and CD8 T lymphocytes which are probably not controlled by TH2 cells. It is likely that such early and stable IFN-γ production strongly favors activation of TH1 cells over TH2 cells. Consistent with this notion, treatment of mice with anti-NK cell antibodies favors TH2 cell differentiation in listeriosis (H. Teixeira, S. H. E. Kaufmann, unpublished). In contrast, evidence for negative regulation of TH1 cells by TH2-like activities in the lepromatous form of leprosy has been obtained (2).

CD8 T CELLS Evidence for an involvement of class I–restricted CD8 T cells in antibacterial immunity was first obtained in murine listeriosis and later confirmed with a variety of other intracellular bacteria including *M. tuberculosis/M. bovis* and *S. typhimurium* (67, 131–140). In vitro, CD8 T cells express specific cytolytic activities and produce IFN-γ (67, 135, 138). Human class I–restricted CD8 T cells with reactivity for intracellular bacteria have been isolated less frequently (141). However, in situ analyses reveal CD8 T cells with cytolytic potential in tuberculosis and in tuberculoid leprosy lesions in which they form an outer mantle surrounding the granulomas (128).

The identification of class I–restricted CD8 T cells with reactivity for intracellular bacteria raises the question of how such antigens are intro-

duced into the class I pathway. Originally it was thought that exogenous antigens remain in the endocytic compartment and hence can only be presented through the MHC class II pathway, whereas newly synthesized antigens have the possibility to associate with MHC class I molecules. However, *L. monocytogenes* is capable of invading the cytoplasmic compartment, with Hly being of major importance for bacterial transition (48). Furthermore, exogenous proteins, when artificially introduced into the cytoplasm, are presented in the context of MHC class I gene products (142). The obvious conclusion, therefore, was that *L. monocytogenes* by virtue of its capacity to enter the cytoplasmic compartment, has access to the class I pathway. Experimental verification of this postulate became possible with the availability of *L. monocytogenes* mutants lacking Hly. In independent studies *L. monocytogenes*-reactive CD8 T cells only responded to targets pulsed with Hly^+ wild-type strain and failed to recognize targets pulsed with Hly^- mutants (143–145). In these experiments, antigen-specific CD8 T cell stimulation depended on the use of viable strains; heat-killed organisms were not presented adequately. However, recognition of Hly^- mutants by CD8 T cells with reactivity for *L. monocytogenes* has also been reported, and in these cases killed microorganisms were recognized, as well (146, 147).

Although the differences between these two types of results cannot be fully explained, it is interesting that the experiments showing dependence on Hly employed relatively short periods of target cell pulsing. In contrast, the experiments suggesting Hly independent class I presentation employed metabolically highly active MP populations and far longer periods of in vitro labelling, which could have allowed for extracellular processing and direct charging of the MHC molecules independent of cytoplasmic processing. Furthermore, Hly independent transition into the cytoplasm by Hly^- mutants cannot be excluded, which—albeit of small degree—could suffice for MHC class I processing (147).

Using the $prfA^-$ *L. monocytogenes* mutant which is defective in expression of a variety of virulence factors but still produces minute amounts of Hly, it was found that this strain is capable of evasive action into the cytoplasm of MP in vitro (147). There, however, it rapidly dies, a finding consistent with its low in vivo virulence. Yet, *L. monocytogenes*–reactive CD8 T cells recognize, equally well if not better, mononuclear phagocytes infected with wild-type *L. monocytogenes* and with $prfA^-$ mutants. Perhaps rapid degradation of this avirulent strain in the cytoplasm provides an ideal source for a multitude of MHC class I antigens. In addition, an unusual intracellular processing pathway from the endosome to the endoplasmic reticulum has been proposed (146). Recent evidence suggests that this type of presentation is apparently not restricted

by conventional MHC class I, but rather involves a conserved class Ib molecule encoded in the M region of the murine MHC (67, 139, 147a,b). N-formylated peptides are presented by this nonclassical MHC gene product. At the moment it seems likely that Hly provides an important, though not exclusive, prerequisite for processing of CD8 T cell antigens.

The general relevance of cytolysin-mediated evasion into the cytoplasm as prerequisite for class I processing of bacterial antigens remains unclear. CD8 T cells have been isolated from *M. tuberculosis*/*M. bovis* and *S. typhimurium*-infected mice (41, 42, 131, 140). Yet, both pathogens replicate inside the endosomal compartment and do not enter the cytoplasm. A possible explanation is that, during the long-lasting persistence in vivo, the endosomal compartment becomes leaky, allowing secreted proteins and/or low mol wt metabolites to enter the class I pathway.

WHICH α/β T CELL CONTRIBUTES TO PROTECTION? The relative contribution of T cell subsets to acquired resistance against intracellular bacteria has been analyzed in two ways: first, by adoptive transfer of T cells from infected donor mice and second by in vivo elimination of T cells by treatment with monoclonal antibodies. In murine tuberculosis, these two approaches have revealed a dominant role for CD4 T cells with some contribution by CD8 T cells (148–152). In contrast, in *L. monocytogenes* infection, CD8 T cells dominate with some contribution by CD4 T cells (8, 133–137, 144, 153). Because both T cell sets express similar biological activities, differential target cell recognition seems to be more important. *M. tuberculosis*/*M. bovis* only resides in MHC class II^+ MP and is highly resistant to intracellular killing; thus protection must rely on MP activation by CD4 T cells. On the other hand, *L. monocytogenes* is rapidly killed by professional phagocytes but also inhabits MHC class II^- nonprofessional phagocytes; hence protection depends on CD8 T cells. Because experimental listeriosis is rapidly overcome after T cell activation, the CD8 > CD4 T cell proportion seems to be adequate for protection in this case, whereas tuberculosis remains chronic, indicating that the CD4 > CD8 T cell proportion is suboptimal and could perhaps profit from stronger CD8 T cell participation.

γ/δ T Lymphocytes

During recent years a huge amount of evidence has accumulated that indicates a role for γ/δ T lymphocytes in antibacterial immunity. First, γ/δ T cells from mice immunized with *M. tuberculosis* respond vigourously to *M. tuberculosis* lysates in vitro (154–156). Second, an extraordinarily high number of thymic and splenic γ/δ T cell hybridomas from mice were found to react with purified protein derivative (PPD) (157, 158). Third, increased

numbers of γ/δ T cells were identified in certain skin lesions of leprosy patients (159, 160). Fourth, marked expansion of γ/δ T lymphocytes from the peripheral blood of normal healthy individuals by in vitro stimulation with *M. tuberculosis* preparations was observed (161–163). These findings indicate γ/δ T cell stimulation by mycobacteria via different but not mutually exclusive pathways: one independent of previous priming and of oligoclonal nature; the second resulting from previous in vivo sensitization and of antigen specific character.

OLIGOCLONAL ACTIVATION Oligoclonal γ/δ T cell activation is particularly prominent with human lymphocytes. Limiting dilution analyses have revealed that as many as one in two γ/δ T cells from the peripheral blood of normal donors are stimulated by *M. tuberculosis* lysates (163). Responsive T cells express the $V_{\gamma 9/\delta 2}$ TCR, although it appears that not all $V_{\gamma 9/\delta 2}$ T cells respond (161, 162). Because cord blood cells and thymocytes are also stimulated, prior in vivo sensitization seems unnecessary (162, 164, 165). Different bacteria and protozoa are stimulatory independent of their intracellular life-style and pathogenicity (161, 166–168). Because $V_{\gamma 9/\delta 2}$ T cells make up a minor proportion of all γ/δ T cells in newborns and subsequently increase to up to 80% of all γ/δ T cells, the in vivo activation by microbial components has been suggested to represent an extrathymic differentiation pathway (165).

γ/δ T cell stimulation by bacteria is reminiscent of the superantigen activity of bacterial toxins for α/β T cells and γ/δ T cells (169, 170). The stimulatory entity for γ/δ T cells in mycobacteria is a low-mass (<3 kDa), protease-resistant molecule, the exact structure of which has not yet been resolved (171). Although it is not easy to envisage how such a small molecule links accessory cells and T lymphocytes, operationally it seems to function as a superantigen. Yet, some evidence has been presented that oligoclonal γ/δ T cell activation by mycobacteria is indirect and results from interleukin induction in accessory cells or α/β T cells which subsequently stimulate γ/δ T cells selectively (164).

Mycobacteria-stimulated γ/δ T cells express various biological functions relevant to defense against intracellular bacteria such as lysis of mycobacteria-pulsed target cells and IFN-γ and TNF secretion (61, 68). Thus, their known biological activity spectrum would argue for participation in antibacterial immunity in a way similar to that of α/β T cells, and the rapid nonspecific activation by microbial components could ensure an effective first line of defense. On the other hand, containment of intracellular bacteria inside host cells may interfere with release of sufficient quantities of the stimulatory components. Recent studies, however, describing the expansion of γ/δ T cells by MP infected with live *M. tuberculosis* indicate

participation of oligoclonally stimulated γ/δ T cells in antibacterial immunity (172).

ANTIGEN-SPECIFIC ACTIVATION γ/δ T cell activation by *M. tuberculosis* is not restricted to the low mol wt component; in addition, larger (> 30 kDa) mol wt preparations, probably containing abundant proteins, are also capable of stimulating γ/δ T cells (171; B. Schoel, S. H. E. Kaufmann, unpublished). The variability between different individuals in responses to these large mol wt components is greater than the responses to the low mol wt fraction. Furthermore, selectivity in the recognition pattern of mycobacteria-stimulated γ/δ T cells has been observed (167).

Modlin et al found increased numbers of γ/δ T cells in skin lesions of leprosy patients following lepromin injection, and in reversal reactions, but not in tuberculoid or lepromatous lesions (160). Thus, γ/δ T cell accumulation was associated with inflammatory rather than with established and chronic lesions. γ/δ T cell lines derived from these lesions responded to *M. leprae* cell wall preparations and PPD. Increased numbers of γ/δ T cells were also observed in necrotic areas of tuberculous lymphadenitis, with the majority of cells concentrated in the vicinity of or inside necrotic regions (173). In contrast, another study reported that lymph node granulomas of tuberculosis patients contained only small numbers of γ/δ T cells (174). Analyses of granulomas in the lung, the major target organ of clinical tuberculosis, need yet to be performed.

When V_δ diversity of γ/δ T cells derived from lepromin reactions was analyzed, the ratio of $V_{\delta 2}$ to $V_{\delta 1}$ chain usage was found to be 2 : 1 compared to 9 : 1 in the peripheral blood of the same individuals (159). Furthermore, both $V_{\delta 1}$ and $V_{\delta 2}$ positive T cells from lesions preferentially used $J^{\delta 1}$ with limited junctional diversity. In contrast, $V_{\gamma 9\delta 2}$ T cells from the peripheral blood after activation by mycobacterial lysates in vitro exhibit extensive junctional diversity (159, 161). These findings indicate a superantigen-independent, *M. leprae*-specific attraction of γ/δ T cells into the lesions.

Accumulation of γ/δ T cells in the lung of mice after aerosol immunization with mycobacterial preparations has been observed (156). Following i.p. infection with *M. bovis* or *L. monocytogenes*, local accumulation in the peritoneal cavity on day 7 or day 3, respectively, was noted (175, 155). In both cases, appearance of γ/δ T cells preceded that of α/β T cells. γ/δ T cells from lymph nodes of *M. bovis*-infected mice as well as those of mice immunized with killed *M. tuberculosis* responded vigorously to mycobacterial antigen preparations (154–156, 176, 175). The γ/δ T cells from *L. monocytogenes*-infected mice produced IFN-γ in response to PPD and hsp 60 but, interestingly, failed to recognize heat-killed *L. monocytogenes* (176). Together, these findings further support antigen-specific

activation of γ/δ T cells during bacterial infections. Furthermore, these and additional data to be discussed later indicate a bias of a subset of γ/δ T cells for hsp 60.

ANTIBACTERIAL PROTECTION Hiromatsu et al treated mice with monoclonal antibodies against the γ/δ TCR or α/β TCR which were then infected with *L. monocytogenes* (176). Animals treated with either antibody suffered from exacerbated listeriosis. Treatment with the anti-γ/δ TCR antibodies transiently increased bacterial numbers in spleens; however, the mice eventually were capable of clearing infection. These data can be taken as evidence that γ/δ T cells are required for optimum protection but are not essential for sterile eradication of bacteria.

L. monocytogenes-infected mice were treated with the anti-γ/δ TCR antibody, GL3,and subsequent proliferative responses to listerial antigens assessed in vitro (177). Marked increase in proliferation was observed. When spleen or lymph node T cells from noninfected, but antibody-treated mice were cultured in vitro in the absence of foreign antigens, they proliferated, produced IL–2, and developed into cytolytic T cells (178). While both CD4 and CD8 α/β T cells contributed to the proliferative response, IL–2 production depended on CD4 α/β T cells and killing on CD8 α/β T cells. Taken together, these data suggest that γ/δ T lymphocytes are involved in the control α/β T cell activation.

Recent experiments with mutant knock-out (KO) mice shed further light on the role of γ/δ T cells in antibacterial immunity (177). Mice deficient in the β- or δ-gene locus were constructed by homologous recombination in the laboratory of Dr. S. Tonegawa by Dr. P. Mombaerts (179). δ-KO mice were infected with *L. monocytogenes*, and subsequently bacterial numbers in spleens and livers of these mice were determined. No differences between δ-KO mice and their normal control littermates were found. In addition, both groups of mice developed comparably strong delayed type hypersensitivity (DTH) reactions against soluble listerial antigens. β-KO mice were capable of controlling primary listeriosis as efficiently as their controls. In contrast, DTH responses to listerial antigens were absent in β-KO mice. Following administration of the γ/δ TCR antibodies, however, increased numbers of *L. monocytogenes* were observed in spleens of β-KO mice. Spleen cells from listeria-infected β-KO mice produced IFNγ. β-KO mice were vaccinated with viable *L. monocytogenes* and then infected with a high, normally lethal, dose of *L. monocytogenes*. While control mice were highly immune against listeriosis and capable of clearing secondary infection rapidly, β-KO mice were only partially immune, i.e. they could reduce but not fully eliminate secondary listeriosis. Vaccinated β-KO mice that had been treated with anti-γ/δ TCR antibodies suffered more strongly

from secondary infection. These data suggest that the function of α/β T cells in protection against primary infection with *L. monocytogenes* can be compensated in mice lacking these T cells with this compensation—at least in part—being due to γ/δ T cells, probably with some contribution by NK cells. In secondary infection, α/β T cells seem to be indispensable, and γ/δ T cells cannot compensate efficiently but they do contribute partially to protection. Thus, these data confirm the essential role of α/β T cells in protective immunity and, moreover, show that γ/δ T cells are not essentially required for clearance of listeriosis, although they contribute to antibacterial immunity. Finally, it is possible that the γ/δ T cells participate in the control of α/β T cell activation during infection.

Local Immunity

Intracellular bacteria do not enter the body directly. Rather, they first pass through mucosal barriers. The lung serves as a major port of entry for the intracellular bacteria, *M. tuberculosis*/*M. bovis* and *L. pneumophila*, while the intestine is used by the agents of food-borne diseases such as *L. monocytogenes* and *S. typhi*/*S. typhimurium*. Remarkable differences between lung and gut exist: the lung can be seen as a virtually sterile organ, whereas the intestine is inhabited by a plethora of microorganisms. In lung alveoli, free mononuclear phagocytes and T cells exist in the external milieu and migrate back into the tissue (180). The intestinal MP and lymphocytes have a more sessile location amidst and beneath epithelial cells (181, 182). Entry into the host via these two different routes, therefore, requires different mechanisms.

M. tuberculosis will only invade the host successfully if a few microbes enclosed within microdroplets are inhaled. Larger droplets are removed by nonspecific bronchial mechanisms. Because of the sterile conditions inside the lung, competition for adhesion to host tissue is a minor problem. The microorganisms are engulfed by alveolar MP in the external milieu and then are either killed without entering the host or translocated into the internal milieu particularly to local lymph nodes.

In contrast, a high degree of microbial competition for adhesion to host cells exists in the gut. Here, successful adhesion and active invasion are the decisive prerequisites for entry into the host. After epithelial invasion, intracellular bacteria such as *L. monocytogenes* will not stay inside enterocytes; rather they leave these cells and are then engulfed by MP which disseminate their intracellular parasites to local lymph nodes and subsequently to internal target organs (183).

Intraepithelial T lymphocytes (IEL) are the first T cells invasive bacteria contact. Although it has been proposed that IEL perform surveillance functions at these important strategic locations, their biological role in

antibacterial defense has not been established (182). Intestinal IEL are interspersed among epithelial cells at a ratio of approximately 15 IEL to 100 enterocytes. In human, approximately 90% IEL use the α/β TCR and 10% the γ/δ TCR, whereas in mice almost 40% are γ/δ TCR$^+$. Recent in vitro studies in the mouse demonstrate that these IEL possess innate effector functions (181, 184, 185). Upon TCR engagement, both α/β and γ/δ IEL express potent cytolytic activities in antibody redirected killer assays and produce IFN-γ (184–186). Active invasion of the intestinal epithelial cells by *L. monocytogenes* triggers IFN-γ secreting potential in γ/δ IEL: the proportion of IFN-γ-secreting γ/δ IEL from mice infected p.o. with *L. monocytogenes* markedly increases, and after restimulation in vitro with *L. monocytogenes*-pulsed stimulator cells γ/δ IEL produce significant levels of IFN-γ (186). In contrast, IFN-γ secretion by α/β T cells in response to *L. monocytogenes* invasion has not been observed. These findings provide first evidence for specific participation of IEL in local defense against bacterial invasion. IFN-γ and possibly other interleukins produced by IEL during bacterial invasion could contribute to local protection in different ways: local stimulation of intestinal MP and enterocytes for antibacterial activity; induction of increased IgA secretion; increased MHC class II expression in enterocytes thereby facilitating interaction with CD4 T cells; repair of enterocytes damaged during invasion.

The Granulomatous Lesion

In general, protective immunity against intracellular bacteria is a local event which is focussed on granulomas. Although inflammatory phagocytes accumulate nonspecifically at the site of bacterial growth, T cells are indispensible for the formation of stable granulomas. At the same time, pathogenesis, at least partially, is the result of T cell responses against infected host cells.

MURINE LISTERIOSIS Spleen and liver are the major target organs of experimental listeriosis of mice. In spleens, *L. monocytogenes* is restricted to mononuclear phagocytes, whereas in livers hepatocytes are infected in addition (1, 7, 46). This has major consequences for overall pathology of listeriosis. Acquisition of resistance against *L. monocytogenes* can be separated into two major phases. During the early phase, T cell–independent mechanisms cause remarkable, though not complete, reduction of bacterial numbers. Phagocytes (both blood-borne monocytes and PNG) are attracted to the site of bacterial growth, there forming inflammatory foci (10, 46). The factors attracting and/or activating inflammatory phagocytes include IFN-γ, TNF, IL–1, IL–8, and C5a (93, 103, 187–189). During the first 24 hr, neutrophils prevail which lyse infected hepatocytes (7). This

allows the release of *L. monocytogenes* from hepatocytes and subsequent killing by more effective phagocytes. At the same time hepatocyte damage results in histopathology. TNF produced by *L. monocytogenes*-infected tissue MP stimulates NK cells to produce large amounts of IFN-γ which, in turn, activates listericidal mechanisms in MP (62).

Later, T cells enter the stage and blood-borne monocytes predominate. Under the additional influence of CD4 T cell–derived interleukins including IFN-γ and CSF, the influx of blood monocytes to the foci accelerates, and structured lesions are formed where MP are subsequently activated by IFN-γ from CD4 and CD8 T cells (66, 67, 187, 190). Lysis of infected hepatocytes by CD8 T cells promotes *L. monocytogenes* uptake by more efficient phagocytes (132, 191). Although CD4 T cells may express cytolytic functions as well, their MHC class II–dependence restricts them to MP (127, 191). At low bacterial numbers, the protective consequences of lysis prevail. With a higher bacterial load, however, lysis causes extensive tissue damage with detrimental consequences for the host as indicated by elevated serum transaminase levels (190). IL–4 may further contribute to histopathology (95). In the absence of CD4 T cells, lesions do not develop (133). Yet, CD8 T cells express potent protection (133). This finding indicates a moderate role for granulomas and suggests that CD8 T cells, in collaboration with inflammatory phagocytes, are sufficient for protective immunity. The role of γ/δ T cells remains incompletely understood. On the one hand they may regulate α/β T cell activation during infection, and on the other hand they can assume effector functions at least in the absence of α/β T cells (175–178). Due to their earlier appearance and broader recognition pattern, they may link the nonspecific NK-cell-dominated phase with the later stage governed by specific α/β T cells.

TUBERCULOSIS The immune mechanisms involved in human tuberculosis are more complex and, generally speaking, are almost exclusively focussed on the granuloma where a labile balance between T cell-mediated immunity and mycobacterial persistence determines the type of granuloma formed and the fate the infection will take (192). Different types of granulomas can be distinguished. The primary lesion begins with an exudative form which then develops into a productive granuloma under the influence of T lymphocytes. Here, T cells are in close contact with MP at various differentiation and activation states. This granuloma has a relatively high cellular turnover: Freshly immigrant monocytes mature into epitheloid cells and multinucleated giant cells which all harbor *M. tuberculosis* organisms. CD4 T lymphocytes predominate which induce tuberculostatic activities in MP via IFN-γ probably in collaboration with TNF and 1,25-dihydroxyvitamin D3 (15, 16). Productive granulomas are surrounded by

a peripheral mantle of CD8 T cells (128). Lysis of incapacitated MP by CD8 and CD4 T cells may directly harm tubercle bacilli (140) and—more importantly—facilitate bacterial release and subsequent uptake by more efficient MP. A role for γ/δ T cells in the early developing granuloma appears likely but incompletely understood, although some evidence for γ/δ T cell participation in MP attraction and accumulation exists (155, 160).

The productive granuloma is highly successful in containing *M. tuberculosis* in distinct foci and in preventing dissemination. Fibrosis and calcification of productive granulomas will further improve containment. Only rarely and in immunodeficient states does the primary lesion break down, leading to dissemination. However, mycobacteria are usually not fully eradicated, and some dormant microorganisms will persist without causing disease. A normal immune status provided, such lesions can persist for decades. In fact, in > 90% of the affected people infection is arrested at this stage without clinical tuberculosis developing (4). In an unknown percentage of individuals the proliferative granuloma can even successfully achieve sterile eradication of *M. tuberculosis* and then heal. In the remaining individuals two forms of granuloma develop which sometimes coexist and transform into each other. In the necrotic lesion, probably under the influence of TNF and cytolytic T cells, the center further necrotizes. Inside such necrotic lesions, the bacteria are still well confined, but tissue injury is extended, thus affecting lung function. Release of bacteria from MP into a necrotic center is primarily lethal for the bacteria because of an insufficient O_2 supply. Alternatively, lytic mechanisms dominate and caseous granulomas develop which are harmful. The cellular detritus in a caseous cavity provides an excellent habitat for *M. tuberculosis* which increases to enormous numbers in this extracellular compartment. At this stage, extensive tissue damage profoundly affects organ function. Moreover, the bacteria are often disseminated by the hematogenic or aerogenic route, thus allowing for infection of other organs and other individuals.

THE ANTIGENS OF INTRACELLULAR BACTERIA

Identification of the relevant antigens from intracellular bacteria is not only of academic interest but also has great practical implications since antigens seen by protective T cells represent subunit vaccine candidates. Protective antigens can be defined as those microbial entities which, under appropriate conditions, induce a protective immune response against the viable pathogen . In humoral immunity, often a hierarchy among numerous antigens exists, and only a few antigens are actively protective. Fre-

quently, protective antigens are virulence factors which mediate adhesion, toxicity, or evasion from phagocytosis so that antibodies against such molecules often provide protection against infection. In the case of intracellular bacteria, the correlation between protective antigens and virulence functions is unclear.

T cells from tuberculosis and leprosy patients as well as from *M. tuberculosis*–infected mice recognize a vast number of bacterial antigens (149, 193–197). A normal bacterium comprises some 1000 proteins which, in principle, can all serve as T cell antigens. Therefore, it would be highly desirable to define biological prerequisites that render microbial proteins antigenic for T lymphocytes and to determine whether all or only a few T cell antigens are equally important for protection. These possibilities are greatly influenced by the accessibility of antigens to MHC presentation. Obviously, structural antigens can only be presented to the immune system after killing and degradation of the microorganism. In contrast, secreted antigens can be processed while intracellular bacteria are still alive and replicating. In fact, metabolically highly active microorganisms would be the best source for secreted antigens. It is, therefore, likely that early antigens, i.e. those presented to T cells soon after infection, are primarily of the secreted type. The more resistant a microbe is to intracellular killing, the more important secreted antigens will be. Consistent with this notion T lymphocytes from patients as well as from *M. tuberculosis*/*M. bovis*-infected mice strongly respond to secreted proteins (149, 193, 196). During chronic infections, however, some microbes will succumb, thereby making structural proteins accessible for antigen processing.

Divergence of microbial proteins into the class I or the class II MHC pathway has a decisive influence on the ensuing T cell response. As discussed above, Hly and probably other cytolysins of *L. monocytogenes* favor class I processing (135, 143–145). Conversely, antigen presentation through the class II MHC pathway is decreased by Hly (198). Balanced release of antigens into the two MHC pathways is an important task for subunit vaccines. Introduction of soluble antigens into the MHC class II pathway is effectively achieved by currently available adjuvants while entry into the MHC class I pathway still poses problems which, however, may be overcome in the future by using new generation adjuvants such as ISCOM (199).

N-formyl-methionine represents a signal sequence for secretion of prokaryotic polypeptides and N-f-met containing peptides can be found in bacterial culture supernatants. Evidence has been obtained that N-formylated peptides of *L. monocytogenes* are presented by murine nonpolymorphic MHC class Ib molecules (147a, b) and *L. monocytogenes* MHC class I nonrestricted specific $CD8^+$ T cells have been shown to

confer antilisterial protection (67). These findings point to the possibility of peptide vaccination against intracellular bacteria independent from MHC polymorphism.

With the large number of potential antigens and given the fact that numerous proteins are shared among various prokaryotes, the possible relevance of cross-reactivity has also to be considered. Cross-reactive T cell responses to antigens shared by various microbes have been identified in several instances with hsp as primary examples (4). Often, these cross-reactive antigens induce a dominant T cell response (200, 201). Because hsp are highly conserved and ubiquitous, T cells with specificity for cross-reactive antigens could be stimulated and constantly boosted through subclinical infection with low virulence bacteria. Upon later infection with highly virulent bacteria, such T cells are rapidly activated to mobilize a first line of specific immune defense (33, 201, 202). Indeed, T cells with specificity for *M. tuberculosis*/*M. bovis* hsp 60 have been identified not only in leprosy and tuberculosis patients but also in healthy individuals without prior contact (125, 201–204). Accordingly, conjugate constructs using mycobacterial hsp 60 and hsp 70 can be used as potent carriers for unrelated vaccine candidates (205, 206).

A striking homology between microbial and mammalian hsp has been observed, and in the case of hsp 60, the bacterial and mammalian cognate share more than 50% sequence homology (33). Thus, hsp 60–reactive T cells could be targeted on autologous hsp, a situation that could be of particular significance for γ/δ T cells (33, 157, 202, 203, 208–210). A focus of T cells on autologous hsp has three potential consequences: First, it allows recognition of stressed and/or damaged host cells, thus promoting immune surveillance functions of hsp 60-reactive T cells early after infection (34). Such a function would be particularly important for sessile T cells such as the IEL (182). Second, recognition of stressed cells could also contribute to histopathology and perhaps even lead to autoimmune disease (33, 202, 211–217). Third, T cells recognizing hsp-derived ligands could perform regulatory functions (178, 216, 218).

Hence, as is the case with antibacterial T cell mechanisms, a single microbial antigen can cause beneficial or detrimental effects for the host.

CONCLUDING REMARKS

In the preceding section I reviewed recent findings relating to the immune response against intracellular bacteria, with emphasis on two quite different infections, murine listeriosis and human tuberculosis. Several conclusions had to be drawn from in vitro experiments and experimental animal studies and, therefore, had to be speculative in part. Yet, without

doubt, interactions between different T-cell subsets and functions are required for optimal protection, with differing emphasis on the participating elements in various types of infection. In addition, it is clear that the same or similar T cell mechanisms also contribute to pathology. Experimental listeriosis is an acute disease which is sterilely eradicated by the T cell response. This seems to represent an optimum means of defense against this pathogen. In contrast one third to one half of the total world population is infected with *M. tuberculosis* despite their having a specific immune response. In the vast majority (>90%) infection will not develop into disease. From the epidemiologic standpoint this reflects a form of highly effective parasitism, while from the immunological perspective it is suboptimal because it does not lead to sterile eradication of the infectious agent and later results in disease in 5% of the infected individuals. At the moment, the reasons for the decision as to whether infection transforms into disease are not completely understood. However, evidence exists that alterations in the host response rather than failure to recognize specific antigens is of importance. Any future vaccination strategy has to consider the already infected and immune population as an important target in which T cell responses have to be improved to induce sterile microbial eradication. Research on the interplay between the immune response and persistent microbial pathogens as well as characterization of the relevant antigens will help us to understand how the immune system performs in a world of "viable antigens" in constant transmutation, an encounter which, after all, was a driving force for its own evolution. This research is also required for the rational development of means that will allow for effective control of major global health problems.

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INTERLEUKIN–10

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Abstract

In the three years since its discovery, the pleiotropic cytokine interleukin–10 (IL–10) has been implicated as an important regulator of the functions of lymphoid and myeloid cells. IL–10's ability to block activation of cytokine synthesis and several accessory cell functions of macrophage renders this cytokine a potent suppressor of the effector functions of macrophages, T cells, and NK cells. In addition, IL–10 likely contributes to regulating proliferation and differentiation of B cells, mast cells, and thymocytes. The Epstein-Barr virus genome encodes a homolog of IL–10 (BCFR1, viral IL–10, vIL–10) which shares many of the cellular cytokine's biological activities and may therefore play a role in the host-virus interaction. This article reviews current studies of IL–10's biological activities and discusses its possible roles in regulation of immune responses.

INTRODUCTION

Activated hemopoietic cells secrete numerous proteins. Several of these proteins, the cytokines, play important roles in the regulation of immune responses by controlling proliferation, differentiation, and the effector functions of immune cells. Most cytokines have more than one biological activity; which activity is the most important probably depends on the local context in which the cytokine is produced. Discovery of the cytokine

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interleukin–10 (IL–10) was based on three different in vitro biological activities.

The cytokine synthesis inhibitory factor (CSIF) activity of IL–10 was discovered by searching for a product of T helper 2 (T_h2) cells that would inhibit proliferation, effector function, and possibly development of T helper 1 (T_h1) cells (1, 2), in a way analogous to inhibition of T_h2 proliferation by IFNγ, a T_h1 cytokine (3, 4). CSIF activity was produced by T_h2 clones and inhibited production of cytokines such as interferon-γ (IFNγ) by T_h1 clones (1). Based on this biological activity, cDNA clones encoding mouse IL–10 (mIL–10) were isolated (5). Subsequently, cDNA clones encoding human IL–10 (hIL–10) were identified by cross-hybridization with the mouse cDNA (6).

Mouse B cell lymphomas expressed an activity that strongly enhanced proliferation of mouse thymocytes in response to IL–2 and IL–4 (7). The same B lymphoma supernatants sustained the viability of mouse mast cell lines in culture and augmented their proliferative response to IL–3 or IL–4 (8). With the use of recombinant IL–10 and neutralizing antibodies (9), these activities were shown to be due to IL–10 (10–12).

This article describes the structure of IL–10, its close relationship to an open reading frame in the Epstein-Barr virus (EBV), and its currently known spectrum of in vitro and in vivo biological activities. Possible roles of IL–10 in the regulation of immune and inflammatory responses are also discussed.

IL–10 PROTEIN AND GENE STRUCTURE

IL–10 cDNA Clones and Protein

The primary structures of mouse (mIL–10) and human IL–10 (hIL–10) were determined by cloning cDNAs encoding the cytokines (5, 6). The mIL–10 and hIL–10 cDNA clones exhibit a high degree of nucleotide sequence homology (>80%) throughout their entire length; the only significant difference is the insertion of a human *Alu* repetitive sequence element in the 3′-untranslated region of the hIL–10 cDNA clone.

The mIL–10 and hIL–10 cDNAs encode very similar (73% amino acid homology) open reading frames (ORF) of 178 amino acids, including hydrophobic leader sequences. Although hIL–10 is active on mouse cells, mIL–10 has not yet been found to cross-react significantly on human cells. Based on its primary structure, IL–10 is a member of the four α-helix bundle family of cytokines (13). hIL–10 is an 18 kDa polypeptide (6) which lacks detectable carbohydrate (6) (P. Trotta, Schering-Plough Research, personal communication), but mIL–10 is N-glycosylated at a site near its N-terminus that is missing from hIL–10. This glycosylation is hetero-

geneous, resulting in a mixture of 17, 19, and 21 kDa species (5, 9). It is not required for biological activity because a mutant lacking the N-linked site is active, and recombinant mIL–10 (rmIL–10) expressed in *Escherichia coli* retains all known biological activities (R. Kastelein & K. Johnson, unpublished).

Both mIL–10 (1; M.W. Bond, unpublished) and rhIL–10 (P. Trotta, Schering-Plough Research, personal communication) are expressed as noncovalent homodimers. The extent to which mIL–10 or hIL–10 monomers are biologically active is not yet certain. mIL–10 and hIL–10 are rapidly inactivated by exposure to acid (pH < 5.5) but appear stable in basic conditions (pH up to 11). The mature N-terminus of mIL–10 was predicted to be Ser19 (5) but was later determined by radiochemical sequencing of rmIL–10 to be Gln22 (J. Wideman, K. W. Moore, R. Kastelein, unpublished). The N-terminus of rhIL–10 is Ser19 (P. Trotta, Schering-Plough Research, personal communication) as predicted (6). mIL–10 and hIL–10 with peptide "tags" of at least eight amino acids at the N-terminus and 21 amino acids at the C-terminus had no detectable loss of biological activity (Y. Liu, A. Ho, D.-H. Hsu, K. W. Moore, unpublished). Recombinant mIL–10 and hIL–10 have been expressed in: COS7 cells, mouse myeloma cells, Chinese hamster ovary (CHO) cells, a baculovirus expression system, and *E. coli*. The biological activities of these rIL–10 proteins are so far indistinguishable.

The IL–10 Gene and Chromosomal Localization

The mIL–10 gene comprises five exons arrayed over ~5.1 kb of DNA (14). The genomic clone itself encodes an expressible mIL–10 protein (S. Wei, K. W. Moore, unpublished). A number of possible transcriptional control sequences were identified in noncoding regions of the mIL–10 sequence, which together resemble the pattern observed for the mouse and human IL–6 genes (15), and as discussed later, several characteristics of IL–10 expression resemble those of IL–6 as well. The mIL–10 and hIL–10 genes are on mouse and human chromosome 1 (14).

Homology to the EBV Gene BCRF1

mIL–10 and hIL–10 exhibit strong DNA and amino acid sequence homology to an open reading frame in the Epstein-Barr virus genome, BCRF1 (5, 6, 16). The homology is confined to the mature protein coding sequence and is not detected in the signal sequence or 5′- and 3′-untranslated sequences. Of the three sequences, mIL–10 and hIL–10 are the more closely related pair at the DNA sequence level (81%), while the DNA sequences encoding the mature hIL–10 and BCRF1 proteins have only 71% homology. hIL–10 and BCRF1 are more closely related in amino acid

sequence (84% identical). A panel of rat anti-hIL–10 antibodies exhibits extensive cross-reactivity with both hIL–10 and vIL–10, but not with mIL–10 (17; J. Abrams, K. W. Moore, J. Silver, unpublished). Unlike the cellular IL–10 gene, the BCRF1 gene contains no introns. These findings suggest that (*a*) the mIL–10 and hIL–10 genes evolved from a common ancestor, (*b*) BCRF1 may represent an ancestral processed, captured cellular cytokine gene, and (*c*) the BCRF1 protein has been constrained to resemble the human cytokine (6, 18).

BCRF1 is expressed in the late phase of the lytic cycle of EBV, but not to a significant level in the latently infected cell lines so far tested (19, 20; D-H. Hsu, K. W. Moore, unpublished). The BCRF1 open reading frame encodes a 17-kDa secreted polypeptide which, like hIL–10, contains little or no carbohydrate (20). As discussed later, BCRF1 displays some of the activities of IL–10 and has thus been designated viral IL–10 (vIL–10). In assays where vIL–10 activity is readily detected, its specific activity appears to be 3–10-fold lower than hIL–10 (18).

Cells Expressing IL–10

mIL–10 is expressed by mouse $CD4^+$ T_h2 clones, at least one $CD8^+$ clone, B lymphomas, T cells, activated mast cell lines, activated macrophages, keratinocytes, and Ly–1 B (B–1) cells (1, 5, 8, 10, 21–25). hIL–10 is expressed by human $CD4^+$ T cells and T_h0, T_h1, and T_h2 T cell clones, by $CD8^+$ T cells and clones (26), monocytes/macrophages, keratinocytes, activated B cells, B lymphomas, and Burkitt lymphoma lines infected with a transforming EBV strain, but not with a nontransforming strain (6, 17, 27–31; J. de Vries, personal communication). Thus, IL–10 is not strictly a T_h2-specific cytokine, and its pattern of expression resembles IL–6 more than IL–4 or IL–5. Like IL–6 but unlike IL–4, IL–10 expression is not inhibited by cyclosporine or FK–506 (32; S. Wang et al, submitted).

IL–10 is produced relatively late following activation of T cells or monocytes/macrophages, compared to other cytokines (17, 21, 26; D. Fiorentino, A. O'Garra, unpublished), which may be an important aspect of IL–10's ability to inhibit T cell and macrophage activation. IL–4 inhibited production of IL–10 by LPS-activated human monocytes (17), and IFNγ inhibited production of IL–10 by murine macrophages (21) and human peripheral blood mononuclear cells (PBMC) (P. Chomarat et al, Schering Plough France, unpublished).

BIOLOGIC ACTIVITIES OF IL–10 IN VITRO

The CSIF Activity of IL–10

Subsets of $CD4^+$ T helper cells characterized by their patterns of cytokine production were described first in mouse (33) and then in human systems

(34–39). T helper 1 (T_h1) cells, which produce IL-2, IFNγ, and lymphotoxin (LT), are effective inducers of delayed-type hypersensitivity (DTH), whereas T helper 2 (T_h2) cells which produce IL-4, IL-5 (and IL-10) provide more effective B cell help. The CSIF activity of IL-10—inhibition of cytokine synthesis by T_h1 clones activated in the presence of APC—was originally detected in supernatants of activated murine T_h2 clones but was not found in supernatants of activated T_h1 clones (1). TGFβ also inhibited IFNγ production by T_h1 clones, but TGFβ or anti-TGFβ antibodies added to the cultures did not affect CSIF activity, and the possibility that it was mediated by TGFβ was excluded (1).

CSIF: INHIBITION OF MONOCYTE-MACROPHAGE ANTIGEN PRESENTING CELL (APC) AND ACCESSORY CELL FUNCTION Evidence suggesting that the inhibitory effect of mIL-10 on mouse T cell cytokine synthesis was indirect (1) was confirmed using purified populations of splenic or peritoneal cell APC to stimulate a T_h1 clone. Antigen-specific T_h1 cytokine synthesis was inhibited when purified mouse macrophages, but not B cells, were used as APC (40). Moreover, stimulation of T_h1 cytokine production by macrophage APC was inhibited by preincubating a macrophage cell line for at least seven hours—two hours may be insufficient (41)—with IL-10. (Further addition of IL-10 at the time of T cell stimulation resulted in more pronounced inhibition; however, this effect was seen only with certain macrophage populations—A. O'Garra, unpublished).

mIL-10 inhibited production of cytokines (at both the mRNA and protein level) by mouse T_h1 clones in response to antigen or soluble anti-CD3 in the presence of macrophage-containing APC (irradiated spleen cells) (1, 40). In contrast, cytokine synthesis by T_h2 clones was not significantly affected. Inhibition of IL-2, IL-3, LT, IFNγ, and granulocyte-macrophage colony stimulating factor (GM-CSF) synthesis by T_h1 cells was most pronounced for IFNγ and IL-3, which are synthesized late after activation.

hIL-10 also inhibited cytokine synthesis. Inhibition by hIL-10 of IFNγ, TNFα, GM-CSF, and LT production by human PBMC stimulated by PHA or anti-CD3 antibodies was observed at the protein and RNA levels (6, 20). hIL-10 inhibited cytokine production by $CD4^+$ human T cell clones when monocytes, but not B cells (EBV-LCL), were used as APC (R. de Waal Malefyt, J. de Vries, unpublished). However, unlike initial results obtained with mouse cells (1, 40), hIL-10 inhibited APC-dependent stimulation of cytokine synthesis by T_h2- and T_h0-like clones, not just T_h1-like clones; and production of "T_h2" cytokines IL-4 and IL-5 was also inhibited. This difference might be partly explained by different stimulation conditions: in the human system peripheral blood monocytes served as

APC, whereas the mouse system utilized irradiated spleen (1, 40). Even so, the ability of a mouse T_h2 clone to produce IL–4 and IL–5 in response to antigen and purified peritoneal macrophage APC was not affected by IL–10 or anti-IL–10 antibodies, although IFNγ production by a mouse T_h0 clone stimulated similarly was inhibited by IL–10 (S. Meding, A. O'Garra, unpublished) Study of a more extensive panel of mouse T_h2 and T_h0 clones may clarify this issue.

However, results from two models of mouse parasite infection offer apparently conflicting evidence on this point. When uncloned $CD4^+$ cells from BALB/c mice infected with *Leishmania major* were restimulated in vitro with *L. major* antigens and bone marrow macrophage APC, IL–10 inhibited production not only of IFNγ, but of IL–4 and IL–3 (F. Powrie, R.L. Coffman, unpublished). In contrast, addition of anti-IL–10 antibodies to an in vitro *Schistosoma mansoni* antigen stimulation resulted in dramatic enhancement of IFNγ production but had no effect on IL–5 levels (42). The results may be reconciled by proposing that the different APC used affected the results or that "T_h2" cytokines can be produced by different T cell subsets (T_h0 and others—43) and that these subsets are differentially sensitive to inhibition by IL–10.

IL–10 also inhibited monocyte/macrophage-dependent cytokine synthesis (IFNγ and TNFα) by human NK cells stimulated by IL–2 (20, 44). IL–10 had no effect on the small amount of IFNγ produced when NK cells were stimulated by IL–2 without accessory cells. IL–2-driven proliferation and generation of lymphokine-activated killer (LAK) activity were not inhibited by IL–10 in this system (44). mIL–10 also inhibited macrophage-dependent cytokine synthesis by mouse NK cells stimulated with either heat-killed *Listeria* (G. J. Bancroft et al, London School of Tropical Health and Hygiene, submitted) or LPS (K. Varkila, R. L. Coffman, personal communication).

IL–10 INHIBITS MONOCYTE/MACROPHAGE-DEPENDENT T CELL PROLIFERATION In the presence of monocyte/macrophage APC, hIL–10 inhibited not only cytokine synthesis, but also proliferation of human T cells and T cell clones (27, 45; R. de Waal Malefyt, J. de Vries, unpublished). The inhibition was monocyte/macrophage-dependent and could be partly, but apparently not completely, overcome by added IL–2 (27, 45, 112).

mIL–10 likewise inhibited proliferation of antigen-stimulated T_h1 clones (1; S. Macatonia, A. O'Garra, submitted) and of mitogen-stimulated mouse $CD4^+$ and $CD8^+$ T cells (41). However, this APC-dependent, inhibitory effect of IL–10 was at least significantly, if not completely, overcome by added IL–2 (1, 41), suggesting that the observed inhibition

of mouse T_h1 clone or mouse T cell proliferation was due in part to inhibition by IL–10 of APC-dependent stimulation of IL–2 production. In addition, a marked decrease in the expression of the p55 IL–2R was observed (41) when stimulation of T cell proliferation was inhibited by IL–10; this decrease was also overcome by addition of IL–2 (41). The ability of mIL–10 to inhibit APC-dependent stimulation of T cell cytokine synthesis and proliferation explains its activity as a TsF (T cell suppressor factor) (22).

Macrophage-dependent proliferation of naive mouse T cells stimulated with either Con A (41) or alloantigen (S. Macatonia, A. O'Garra, submitted) was significantly inhibited by IL–10, as was accessory cell-dependent, mitogen-induced proliferation of human T cells from PBMC (45; R. de Waal Malefyt, unpublished). In each system, both $CD4^+$ and $CD8^+$ T cell proliferation were affected (41, 45). Inhibition of both proliferation and cytokine production was also observed in a primary MLR with allogeneic human monocytes (but not EBV-LCL) as stimulator cells, and purified human T cells as responders (112)

EFFECTS ON DENDRITIC CELL APC In contrast to macrophage/monocyte dependent T cell proliferation, IL–10 did not inhibit dendritic cell–induced proliferation of either mouse T_h1 clones or $CD4^+$ T cells in a primary MLR (S. Macatonia, A. O'Garra, submitted), or $CD4^+$ T cells in response to mitogen (41). However, IL–10 did inhibit dendritic cell–dependent stimulation of cytokine (IFNγ) production by T_h1 cells in response to antigen and by $CD4^+$ cells in an MLR, despite a lack of effect on IL–2 production in the latter system (S. Macatonia, A. O'Garra, submitted). The contrasting effects of IL–10 on dendritic cell–induced IFNγ production and proliferation of $CD4^+$ T cells suggest different pathways for stimulation of IFNγ synthesis and proliferation. Alternatively, IL–2 production may occur earlier than IFNγ and thus be less susceptible to IL–10 action (1; S. Macatonia, A. O'Garra, submitted).

MECHANISM OF INHIBITION OF ACCESSORY CELL/APC FUNCTION In contrast to its selective effects on dendritic cells, IL–10's inhibition of macrophage/monocyte APC function appears to affect most aspects of T cell activation, including proliferation and cytokine synthesis (27, 40, 41, 45). The mechanism by which this occurs is not yet completely understood. Certainly one important aspect is that hIL–10 downregulated both constitutive and IFNγ-induced expression of HLA DR/DP and HLA/DQ expression by human monocytes, but not by EBV-LCL (27). Because antigen/APC-induced Ca^{2+} fluxes in human T cell clones were diminished when monocytes preincubated with IL–10 were used as APC, it was proposed that downregulation of monocyte/macrophage class II MHC

expression by IL–10 prevented rather than suppressed T cell activation (27). A similar inhibitory effect of mIL–10 on IFNγ-induced expression of class II MHC antigens on peritoneal murine macrophages elicited by polyacrylamide beads was also observed (M. Stein, S. Gordon, Oxford University, personal communication). In contrast, downregulation of class II MHC antigen expression on either resident murine peritoneal macrophages (M. Stein, S. Gordon, personal communication; A. O'Garra, unpublished) or a mouse macrophage line that responds to IL–10 (40) was not observed, although it may have been obscured by slower turnover rates of these cell surface molecules.

However, inhibition of class II MHC expression alone may not be sufficient to explain the CSIF activity of IL–10, because CSIF activity is observed in stimulations that are independent of antigen/MHC, yet still require macrophage accessory cells. IL–10 suppressed monocyte/macrophage-dependent mouse T cell (40) and human NK cell (44) cytokine synthesis stimulated by IL–2, and IL–10 also inhibited macrophage-dependent mouse NK cell IFNγ synthesis (G. J. Bancroft, personal communication; K. Varkila, R. L. Coffman, personal communication).

As discussed below, IL–10 profoundly inhibited mitogen-induced monokine production by both mouse and human macrophages/monocytes (17, 21). However, current studies of both mouse and human cells suggest that IL–10's effects on macrophage/monocyte APC function are not due to inhibited production of a soluble costimulator(s) required for optimal Th cell activation (17, 21, 41). Furthermore, supernatants from IL–10–treated, activated macrophages did not directly inhibit Th cell cytokine synthesis or proliferation (17, 21). Evidence against such an inhibitor's acting over a short range or having unusual lability was obtained from cocultures of macrophages with limiting numbers of dendritic cells (41) or B cells (21), in which only inhibition of macrophage APC function was achieved with IL–10.

Current evidence thus favors the idea that IL–10 inhibits production or function of a membrane-bound costimulator required for activation of NK cells and T_h1 cells. A possible candidate is B7/BB1 (46–48), the ligand for T cell antigens CD28 and CTLA–4. Current studies are addressing the possibility that IL–10 interferes with B7/BB1 function or expression on macrophages.

Effects on Monocyte/Macrophage Function

IL–10 has pronounced effects on the morphology, phenotype, cytokine production, and function of mouse and human monocytes/macrophages.

IL–10 induced rounding up of the cells and reduced adherence in cultures of purified murine peritoneal macrophages (21) and human monocytes (R. de Waal Malefyt, unpublished).

IL–10 also induced changes in the expression of cell surface molecules on human monocytes. As already discussed, IL–10 inhibited class II MHC expression on human monocytes (27). Constitutive and IL–4- or IFNγ-induced expression of HLA-DR/DP and DQ were inhibited by hIL–10 and vIL–10. In addition, monocytes preincubated with IL–10 were refractory to subsequent induction of class II MHC expression by IL–4 or IFNγ. Furthermore, endogenously produced IL–10 inhibited class II MHC expression by human monocytes following activation by LPS (17, 27). IL–10 did not affect the expression of CD11abc/CD18, CD14, CD54 (ICAM–1) or CD58 (LFA–3) on human monocytes either alone or following activation by LPS, IL–4, or IFNγ, and even stimulated expression of FcγRI (CD64) on human monocytes, which correlated with enhanced FcγRI-mediated capacity to lyse anti-D-coated human Rh+ erythocytes (ADCC activity) (49). In addition, IL–10 prevented downregulation of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) expression by human monocytes during culture of these cells with IL–4 (49).

Production of monokines following activation of human and mouse monocytes/macrophages is strongly inhibited by IL–10 (17, 21, 30, 50). hIL–10 inhibited synthesis of IL–1α, IL–1β, IL–6, IL–8, TNFα, GM-CSF, and G-CSF by human monocytes following activation by LPS or LPS+IFNγ at both mRNA and protein levels (17). Similarly, mIL–10 inhibited production and mRNA levels of IL–1, IL–6, and TNFα by murine macrophage cell lines and peritoneal macrophages activated by LPS or LPS and IFNγ (40, 50). These in vitro effects of IL–10 on monokine production have also been demonstrated in an in vivo model, LPS-induced (endotoxin) shock (M. Howard, personal communication). BALB/c mice given 1–10 μg of IL–10 concomitantly with a lethal dose of LPS were protected from death.

Human monocytes and mouse macrophages can produce IL–10 following activation, and this endogenous IL–10 also inhibits the production of monokines (17, 21). hIL–10 inhibited its own production by LPS-activated monocytes, indicating that expression of IL–10 may be regulated by negative feedback in these cells (17). Thus, endogenously produced IL–10 has autoregulatory activities, not only on monokine production but also on class II MHC expression by LPS-activated monocytes (17).

hIL–10 inhibited production of pro-inflammatory cytokines but in contrast enhanced production of the IL–1 receptor antagonist (E. Vannier, R. de Waal Malefyt, unpublished), which has anti-inflammatory activities (51).

mIL–10 inhibited production of reactive oxygen intermediates (H_2O_2) and reactive nitrogen intermediates (NO) by macrophages following activation by IFNγ (50, 52, 53). IL–10 inhibited NO production if it was present before or during the first 12 hr of IFNγ activation, i.e. the kinetics were similar to those observed for the CSIF activity of IL–10 (6, 21, 40). The inhibition was reversible by a later IFNγ stimulation in the absence of IL–10 (54). Production of NO by IFNγ-activated macrophages is dependent on endogenous TNFα, and inhibition by IL–10 of NO production by IFNγ-activated monocytes was substantially due to inhibition of TNFα production (54).

These results collectively indicate that IL–10 exerts strong anti-inflammatory activities and has thus been termed a "macrophage deactivating factor" (50).

Direct Effects of IL–10 on T Cells/Thymocytes

Although IL–10 affects indirectly many T cell functions through its action on APC, in vitro experiments suggest the possibility of a direct role in the growth, differentiation, activation, and function of T cells and thymocytes. mIL–10 has B cell–derived T cell growth factor activity, inducing proliferation of murine thymocytes in the presence of IL–2 and IL–4 (7, 10). Although mIL–10 alone has no growth factor activity on fetal thymocytes, adult thymocytes or T cells, in the presence of IL–2 and IL–4, it is a cofactor for proliferation of day 15 fetal thymocytes, adult CD3-CD4-CD8$^-$ (triple negative), CD4$^-$CD8$^-$ (double negative), CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ (single positive) thymocytes as well as single positive T cells from spleen and lymph node (7, 10). In addition, mIL–10 enhances proliferation of adult thymocytes in combination with either IL–2, IL–4, or IL–7 singly. Culture of adult double negative and triple negative thymocytes in a combination of IL–2, IL–4, and IL–10 resulted in strong induction or enhancement of CD3 expression measured on day 4 (10). A high proportion of these CD3$^+$ cells expressed the TCRγδ/CD3 complex. Except for CD4$^+$CD8$^+$ thymocytes, all thymocyte and T cell subpopulations, including day 15 fetal thymocytes, were able to produce IL–10 following polyclonal activation (10). These results collectively suggest a possible role for IL–10 in T cell development or differentiation. However, as with many other cytokine functions, this role of IL–10 may not be unique or essential, since initial studies of IL–10 gene-deleted (IL–10 Δ) mice have not yet revealed striking abnormalities in the peripheral T cell compartment (R. Kuhn, K. Rajewsky, W. Mueller, University of Cologne, personal communication).

So far, analogous activities of hIL–10 on human thymocytes have not been detected. hIL–10 has not yet been found to affect proliferation or

phenotype of total human thymocytes or thymocyte subsets, either alone or in combination with IL–2, IL–3, IL–4, IL–7, or GM-CSF, but it did enhance the viability of triple negative thymocytes (T. Hori, H. Spits, unpublished).

Enhancing effects of mIL–10 on the growth of $CD4^-CD8^+$ spleen T cells were also detected as increases in cytotoxic cell precursor frequency and clone size following activation of $CD4^-CD8^+$ single cell cultures in the presence of ConA and IL–2 (55; W. F. Chen, A. Zlotnik, submitted). In addition to the enhancing effects on proliferation, mIL–10 augmented the cytotoxic activity of $CD4^-CD8^+$ effector cells in a lectin-dependent cytotoxicity assay. This was confirmed at the single cell level: mIL–10 enhanced the frequency of murine cytotoxic T cell precursors generated from anti-CD3 activated $CD4^-CD8^+$ T cells and markedly increased the size of the generated clones and their cytolytic activity (55). mIL–10 was required during the entire culture period and did not induce LAK activity in these purified $CD4^-CD8^+$ T cells.

Mouse $CD8^+$ cells expanded in the presence of IL–2, IL–10, and several other cytokines acquired a potential to produce more IFNγ upon subsequent stimulation, compared to cells grown in the same cytokine combination without IL–10 ((55); W.F. Chen, A. Zlotnik, submitted). At first this appears a puzzling contrast to IL–10's inhibitory effect (CSIF activity) on IFNγ synthesis in stimulations involving accessory cells. However, the former activity reflects a role of IL–10 in T cell differentiation, while CSIF activity is quite separately an effect of IL–10 on the ability of the macrophage to function as APC (27, 40). In fact, IFNγ synthesis by mouse $CD8^+$ T cell clones in response to alloantigen presented by irradiated spleen APC was inhibited by IL–10 (T. A. T. Fong, T. R. Mosmann, unpublished).

Thus mIL–10 appears to enhance T cell proliferation in some systems but inhibits T cell proliferation which is dependent on macrophage APC for IL–2 production. Human T cells also exhibited inhibition of proliferation due to inhibition of macrophage APC function, but in contrast to the mouse systems, IL–10 also has direct inhibitory effects on the proliferation of human peripheral blood T cells and $CD4^+$ T cell clones (R. de Waal Malefyt, J. de Vries, submitted) in a macrophage/monocyte-free system. In the presence of mouse L cells expressing human CD32/FcγRII, proliferation of $CD4^+$ human T_h0, T_h1, and T_h2 T cell clones and of peripheral blood T cells induced by anti-CD3 or anti-CD2 mAbs was inhibited by hIL–10. hIL–10 acted directly on the human T cells in this system, since mIL–10, which is species-specific, was not active. Coexpression on CD32-L cells of additional transfected accessory molecules ICAM–1, LFA–3, or B7—the ligands of LFA–1, CD2, and CD28

respectively on the T cell—did not reverse inhibition of proliferation by hIL–10. In addition, hIL–10 had no effect on cell surface expression of CD2, CD3, CD4, CD28, and LFA–1 by T cells, nor did it inhibit expression or induction of IL–2Ra and IL–2Rb chains (R. de Waal Malefyt, J. de Vries, submitted).

However, low concentrations of IL–2, but not IL–4, could completely reverse the inhibition of proliferation by hIL–10 in this system, a result contrasting with findings obtained with monocyte/macrophage APC (27, 41). Thus, this direct inhibition of T cell proliferation was due principally to an inhibition of IL–2 production, which could also be detected at the mRNA level. In contrast, production and mRNA levels of IL–4, IL–5, IFNγ, and GM-CSF were not affected by hIL–10 in this monocyte-free system. Similar results were observed following activation of T cell clones by anti-CD3 mAbs and TPA in the absence of accessory cells. These results argue for a difference(s) in the signal transduction pathway for production of IL–2 and suggest that IL–10/IL–10R interactions on $CD4^+$ human T cell clones and peripheral blood T cells may interfere selectively with activation processes leading to IL–2 synthesis.

hIL–10 also directly affected another function of human $CD8^+$ T cells (T. Jinquan, C. G. Larsen, B. Gesser, K. Matsushima, K. Thestrup-Petersen, University of Aarhus, submitted). hIL–10 was found to be chemotactic for peripheral blood $CD8^+$ T cells but not for $CD4^+$ T cells. In addition, hIL–10 inhibited the chemotactic response of purified $CD4^+$ T cells, but not that of $CD8^+$ T cells, towards IL–8.

IL–10 and Mouse Mast Cells

mIL–10 and hIL–10 extended the viability of mouse mast cell lines in vitro and synergized with mIL–3 and/or mIL–4 to stimulate proliferation of these cell lines (6, 11, 12) in culture. In addition, IL–10 significantly augments both the number and size of mast cell colonies grown from mesenteric lymph node precursors isolated from nematode-infected mice in the presence of IL–3 and IL–4 in vitro. Mast cells in these colonies had the characteristics of mucosal-like mast cells (11). IL–10 also reversibly induced transcription of the mast cell neutral protease gene MMCP–2 in bone marrow-derived mast cells developed in the presence of IL–3 (56). IL–10 therefore appears to be one of a number of cytokines capable of affecting mast cell development and differentiation. However, so far similar activities are not yet demonstrated in human mast cell development (T. Ishizaka, La Jolla Institute, personal communication).

IL–10 and B Lymphocytes

MOUSE B CELLS mIL–10 and hIL–10 enhanced expression of class II MHC on small, dense resting mouse splenic B cells (57). In this respect the

activity of IL–10 is similar to IL–4. However, unlike IL–4 (58; G. Nunez, University of Michigan, personal communication) IL–10 neither detectably affected expression of CD23 (FcεRII) on these cells (57), nor did it induce expression of *bcl*–2 in B cells (G. Nunez, personal communication). IL–10 is also a viability maintenance factor for mouse B cells in vitro (57). mIL–10 did not induce proliferation of resting B cells and LPS-activated B cells, but an effect of mIL–10 on immunoglobulin production by mouse B cells in vitro was reported (59).

IL–10 did not induce class II MHC antigens on B cells from male CBA/N × DBA/2 F1 X-linked immunodeficient (XID) mice, even though this response of the immunodeficient B cells to IL–4 was unchanged (57). However, IL–10 still sustained viability of XID B cells in culture (N. Go, M. Howard, unpublished). These observations suggest that IL–10 may act through at least two distinct receptor-signal transduction systems in mouse B cells, one of which transduces the signal for upregulation of class II MHC expression, an idea also supported by studies of vIL–10 biological activities, as discussed later.

The observed production of substantial amounts of IL–10 by mouse $Ly1^+$ B (B–1) cells (8, 24), together with data supporting a self-renewing capacity of $Ly1^+$ B cells (60), suggested a possible role for IL–10 as a growth factor for mouse B cells. This possibility prompted Ishida et al to treat mice with neutralizing anti-IL–10 antibodies from birth to adulthood (61). These experiments are discussed below, but the results did not clearly demonstrate a role for IL–10 as an autocrine growth factor for $Ly1^+$ B cells.

HUMAN B CELLS Studies of IL–10's effects on human B cells have been reported (62, 63). Unlike results obtained with mouse splenic B cells, hIL–10 did not alter expression of MHC class II antigens on purified human tonsil B cells. This result may be related to already high levels of constitutive class II MHC expression by human tonsil B lymphocytes.

hIL–10 costimulated B lymphocyte proliferation (3H-thymidine incorporation and increased cell number) induced by cross-linking surface Ig with immobilized anti-IgM antibody or by culture with *Staphylococcus aureus* Cowan (SAC) for 48 hr. The costimulatory activity was of lower magnitude than that exhibited by either IL–2 or IL–4. However, the costimulatory effect of IL–10 on B cell proliferation was substantially greater when B cells were stimulated by cross-linking of their CD40 antigen with anti-CD40 antibody and mouse L cells expressing human FcγRII/CD32 (62, 64). IL–10 and IL–4 alone had comparable activity, but their combination resulted in at least a ten-fold expansion of the number of viable B cells during a ten day culture.

IL–10 was also a differentiation factor for SAC– and anti-CD40-activated B cells (62), inducing them to produce large amounts of IgM, IgG, and IgA. This activity was due all or in part to the ability of IL–10 to augment substantially the total number of Ig-producing cells in the cultures, and the activity was significantly antagonized by IL–4. The presence of TGFβ further enhanced both secreted IgA and the number of IgA-producing cells (63). It is yet unclear whether the observed IgG and IgA production reflects either a combination of isotype switching by naive IgD^+IgM^+ B cells or differentiation of already committed B cells, although both IgD^+ and IgD– B cells were inducible for Ig secretion in this system (63). In a different culture system, IL–10 inhibited IL–4–induced production of IgE by PBMC (65; J. Punnonen, J. de Vries, unpublished). However, IgE production by purified B cells activated with IL–4 and anti-CD40 mAb or $CD4^+$ T cell clones was unaffected, suggesting that the effect observed with PBL was indirect, possibly mediated by monocytes. These prominent effects of IL–4 and IL–10 on proliferation and differentiation of activated human B cells suggest that the two cytokines may account for much of the superior ability of T cells expressing them to provide help for B cell responses.

Biological Activities of BCRF1/vIL–10

VIL–10 IS A VIRAL CYTOKINE vIL–10 shares many of the activities of cellular IL–10. vIL–10 exhibits CSIF activity on both human and mouse cells (6, 17, 20, 27, 44), although as noted earlier, its specific activity is consistently lower than hIL–10. vIL–10 also inhibited IFNγ synthesis stimulated by IL–2 in human NK cells (20, 44). vIL–10 is also a macrophage deactivating factor, inhibiting superoxide anion production following activation of monocytes by IFNγ, GM-CSF, or M-CSF (66). vIL–10 exhibited several of the activities on mouse and human B cells which have been characterized for mIL–10 and hIL–10. Although it did not detectably induce class II MHC expression on mouse B cells, vIL–10 did sustain viability of mouse B cells in culture (57). vIL–10 also has all known activities of hIL–10 on human B lymphocytes: it costimulated proliferation of human B lymphocytes activated with anti-Ig or anti-CD40 and was a potent inducer of immunoglobulin secretion (62, 63).

However, the abilities of vIL–10 to stimulate class II MHC induction on mouse B cells (57) or costimulate proliferation of mouse thymocytes (10) and mouse mast cells (6, 11) were by comparison substantially reduced or absent. Attempts to demonstrate an antagonistic effect of vIL–10 on hIL–10 activity in these systems were so far unsucessful (P. Vieira, K. W. Moore, unpublished). Possibly, these results indicate the existence of at

least two types of receptor/signal transduction system for mIL–10 (as noted earlier—57), one of which interacts poorly with vIL–10.

It is important to note that these particular activities were so far characterized only on mouse cells, and it remains to be determined if either hIL–10 or vIL–10 has similar effect(s) on human cells. If such differences are ultimately found also on human cells, then vIL–10 may be considered a naturally occurring partial agonist (67, 68) of hIL–10. The activities in question may not have been subjected to strong selection during evolution or may be deleterious to virus survival. Alternatively, because these activities have not yet been demonstrated in human systems, it is possible that the IL–10 receptor involved may function in the mouse but play little or no role in human cells.

VIL–10 AND THE LIFE CYCLE OF EBV The observation of such strong homology between an EBV and cellular cytokine gene, the products of which both have biological activity on human cells, raises the issue of the advantage conferred on the virus by vIL–10. The CSIF activity of vIL–10 would very likely benefit EBV. Both T cells and NK/LGL cells inhibit viral infection and transformation of B cells (69–71). IFNγ plays an important role in this inhibitory activity (72–74). The BCRF1 gene is transcribed in the late phase of the EBV lytic cycle (19; D.-H. Hsu, K. W. Moore, unpublished), when viral proteins and virus particles are produced. During the period when such virus could infect fresh B lymphocytes, vIL–10 may inhibit virus-induced T or NK cell activation.

Conservation of the activities of hIL–10 on B cells implies a possible role in the ability of EBV to transform human B cells. Even though transformation of B cells by EBV involves a number of other viral proteins (75, 76), the ability of vIL–10 (and hIL–10) to promote growth and differentiation of human B cells suggests a role for either or both cytokines in this process.

In addition, the ability of vIL–10 to enhance differentiation of Ig-secreting cells suggests the possibility that, by enhancing secretion of anti-EBV antibodies, it could thereby promote Fc receptor–mediated uptake of virus into susceptible cells (75) which might otherwise be infected at much lower efficiency. A BCRF1-deleted EBV has been prepared (E. Kieff, Harvard University, personal communication), and although initial experiments revealed no clearly altered ability to transform B cells in vitro, additional studies using PBMC from EBV+ and EBV− donors may provide insight into the role of vIL–10 in the EBV life cycle.

Expression of vIL–10 or induced expression of hIL–10 in infected B cells (6) may play a role in any of several proliferative and immune response aberrations seen in EBV-associated disorders (75, 76), among which are

infectious mononucleosis, chronic fatigue syndrome, and X-linked immunoproliferative syndrome (Duncan syndrome) (77). Exploitation of gene products which mimic cytokines, their receptors (78, 79), or other molecules (80, 81) may be one means for circumventing or delaying host defenses.

REGULATION OF IMMUNE AND INFLAMMATORY REACTIONS BY IL–10

IL–10 and Regulation of T_h2 Phenotype Development

That IL–10 inhibits T_h1-related immune functions is clear, but does it play a role in regulating development of a dominant T_h2 response? IL–10's ability to inhibit stimulation of T cell cytokine synthesis suggested that this cytokine might play a role in $CD4^+$ T cell subset differentiation by affecting stimulations requiring macrophage APC. However, dendritic cells are believed to be the principal APC involved in initiation of immune responses, and because IL–10 does not inhibit dendritic cell–induced T cell *proliferation* (41; S. Macatonia, A. O'Garra, submitted), it is unlikely that it suppresses clonal expansion during initiation of an immune response. However, inhibition of dendritic cell–induced IFNγ production by $CD4^+$ T cells does suggest that IL–10 could thereby prevent the immune system from sustaining development of a T_h1 response.

Nonetheless, the bulk of present evidence favors IL–4, rather than IL–10 as the cytokine playing the more important role in T_h2 cell development. In both polyclonal and antigen stimulations, IL–4 promotes development of T_h2 cells and inhibits development of cells with a T_h1 cytokine secretion phenotype (82–86). IL–4 specifically protects T_h2 cells from glucocorticoid-induced apoptosis (87), suggesting that it may also protect T_h2 cells from programmed cell death. In vivo, IL–4 promotes development of a T_h2 response against *Leishmania major* infection (88), although it does not render the mouse susceptible to the parasite. Anti-IL–4 antibody, when given before or within the first week after *Leishmania* infection, can render a susceptible mouse strain resistant to the parasite (89), but anti-IL–10 does not have this effect (90). Using an $\alpha\beta$-TCR transgenic mouse line as a source of large numbers of cells that could be stimulated with a single antigen in vitro, Hsieh et al (91) showed that the presence of IL–4 promoted development of the cultures toward a T_h2 pattern of cytokine expression; a similar activity was not demonstrated for IL–10. However, neutralization (by antibody) of endogenous IL–10 expressed in the cultures did promote differentiation toward T_h1-like cytokine expression. Thus, current data favor the idea that IL–4 promotes differentiation of T_h2 cells, while IL–10

can inhibit differentiation of T_h1 cells, possibly by blocking synthesis of IFNγ and/or antagonizing its activating effects on APC.

IL–10 Expression and Function in Model Parasite, Mycobacterial, and Retroviral Infections

In view of IL–10's activity as an inhibitor of "T_h1" cytokine synthesis and macrophage functions (17, 21, 27, 30, 40, 44, 50, 52, 53), several studies have examined expression and activity of IL–10 in parasite and mycobacterial infections. In most of these systems, substantial evidence already existed implicating "T_h1" immunity and activated macrophages as key components of effective host defense, and "T_h2" immunity as being ineffective or even harmful to the host (36, 90, 92–96).

PARASITE INFECTIONS In progressive murine leishmaniasis and in mice infected with *Schistosoma mansoni*, a strong T_h2-like immune response occurs, with concomitant suppression of the T_h1-like response. The strong T_h2 response in *Schistosoma mansoni* infection can even exert a bystander effect, altering the response to an unrelated antigen (97). As expected, given IL–10's expression by T_h2 cells in vitro, expression of IL–10 mRNA (98) and protein (42), respectively, were elevated in these situations. IL–10 mRNA detected in *Leishmania*-infected BALB/c (susceptible) mice (98) was found predominantly in $CD4^+$ cells and non-T/non-B cells (presumably macrophages—17, 21). Furthermore, neutralization of IL–10 expressed in vitro by cells from schistosome-infected mice restored the capacity of those cells to produce the macrophage-activating cytokine IFNγ in response to stimulation by parasite antigen (42), and also partially enhanced the ability of cells from infected mice to produce IFNγ in response to a nonparasite antigen (97). These observations suggest that IL–10 plays an important role in suppression of T_h1 immune function(s) in *Schistosoma mansoni* infection.

Subsequent studies of murine *Toxoplasma gondii* and *Schistosoma mansoni* infection revealed in addition that IFNγ-dependent killing of these two parasites by macrophages, which occurs via a nitric oxide (NO)-dependent mechanism (92, 93), was inhibited by IL–10 (52). IL–10 shared this activity with IL–4 and TGFβ, and any combination of two of these three cytokines at suboptimal concentrations exhibited a synergistically enhanced inhibition of killing (54). These findings correlated with the observation that IL–10 inhibited NO production by IFNγ-activated macrophages (50, 52, 53). Inhibition by IL–10 of NO production and parasite killing appears to be due substantially to inhibition of TNFα production by IL–10, since exogenous TNFα reportedly blocked the IL–10 effect (93). In view of these results, one can conclude that IL–10 not

only inhibits T cell production of the macrophage activating cytokine IFNγ, but it directly inhibits macrophage activation as well.

Similar findings were reported in a study of murine *Trypanosoma cruzi* infection (99). These investigators identified susceptible (B6) and resistant (B6D2) mouse strains and showed that resistant mice could be rendered susceptible by treatment with anti-IFNγ antibody. Moreover, spleen cells from infected B6 mice produced IL–10 in vitro, while B6D2 spleen cells did not. Reciprocal expression of IL–10 and IFNγ was also shown in CBA/N mice infected with T. cruzi (P. Minoprio et al., unpublished). Female mice were susceptible and showed elevated IL–10 and diminished IFNγ production, while males carrying the XID gene were resistant and expressed high IFNγ and low IL–10 levels. IL–10 also suppressed the ability of IFNγ-stimulated macrophages to inhibit intracellular replication of the parasite (99).

MYCOBACTERIAL INFECTION Like several of the parasites discussed above, mycobacteria are intracellular pathogens, growing inside macrophages in vivo. Studies of *Mycobacterium leprae* infection have suggested that responses to infection can likewise fall into T_h1-like (tuberculoid and "reversal reactions') and T_h2-like (lepromatous and erythema nodosum leprosum—"ENL"—reactions) forms (36, 94, 95). Tuberculoid leprosy is generally regarded as the "resistant" form of the disease, while lepromatous leprosy is considered the "susceptible" form. T_h2 cytokine mRNAs, including IL–10, are more prominently expressed in the lepromatous state, while those cytokine mRNAs expected to be expressed by T_h1 cells and activated macrophages dominate in tuberculoid leprosy lesions (29). *M. leprae* was found to trigger IL–10 release from PBMC of lepromatous patients and healthy donors, and this IL–10 inhibited proliferation and production of TNFα, IFNγ, and GM-CSF in response to the *M. leprae* stimulus (P. A. Sieling, R. Modlin, UCLA, submitted). The long-standing observation that $CD8^+$ T cells predominate in infiltrates in lepromatous leprosy lesions (100, 101) is also of interest in view of the finding that IL–10 is chemotactic for $CD8^+$ T cells but inhibits $CD4^+$ T cell migration (T. Jinquan, C. G. Larsen, B. Gesser, K. Matsushima, K. Thurstrup-Petersen, submitted). In the so-called "reactional states" (reversal reactions and ENL), T_h1 cytokine mRNA and T_h2 cytokine mRNA levels (including IL–10), respectively, were elevated (96).

Furthermore, lipoarabinomannan, a cell wall component of *Mycobacterium tuberculosis*, induced synthesis of monokines, including IL–10, by human macrophages (102). IL–10 was found to increase permissiveness of human macrophages in vitro for growth of *Mycobacterium avium*, and impaired the ability of infected macrophages to kill *M. avium* in response

to TNFα or GM-CSF (L. Bermudez, Kuzell Institute, personal communication).

RETROVIRUS INFECTION A mouse model of retrovirus-induced immunodeficiency, LP-BM5 (MuLV)-induced "MAIDS," has also revealed characteristics of strong T_h2 and suppressed T_h1 immunity (93). In susceptible mouse strains, virus infection is accompanied by prompt polyclonal T cell activation, probably by a virus-associated superantigen, followed by $CD4^+$ cell-dependent B cell proliferation and differentiation, and immune deficiency (103–105). In vitro cytokine expression by cells from virus-infected mice progressed within the first four weeks of infection from a mixed T_h1/T_h2 ("T_h0") pattern to a T_h2 pattern, characterized by high expression of IL–4, IL–6, and IL–10, and low expression of IL–2 and IFNγ (106). However, in this system evidence for a functional role of IL–10 in development of pathology has not yet been described.

Effects of Anti-IL–10 Antibodies In Vivo

Ly–1 B (B–1) cells comprise about 2% of the B cells of an adult mouse and are found mostly in the peritoneal and pleural cavities. They appear to be self-replenishing and have a number of other properties that distinguish them from conventional B cells (60). The finding that IL–10 was produced by Ly–1 B (B–1) cells (8, 24) prompted speculation that IL–10 might be an autocrine or paracrine growth factor for these cells. To test this idea, mice were treated continuously from birth to 8 weeks with a monoclonal rat IgM antibody that specifically neutralizes IL–10 (61). This treatment resulted in depletion of Ly–1 B cells in the peritoneal cavity. The effect was accompanied by a striking decrease in serum IgM and IgA antibodies, and an inability of the animals to mount antibody responses to α1,3-dextran or phosphorylcholine, properties previously attributed to the Ly–1 B cell compartment (60). Anti-IL–10 treatment did not alter the number, phenotype, or in vitro responsiveness of conventional splenic B cells to mitogens or to the thymus-dependent antigen trinitrophenyl-keyhole limpet hemocyanin. These results were later reproduced with an anti-IL–10 of the IgG isotype (H. Ishida, M. Howard, unpublished).

The depletion of peritoneal Ly–1 B cells was reversible upon withdrawal of anti-IL–10 and was not directly caused by absence of IL–10, since coadministration of anti-IFNγ with anti-IL–10 reversed Ly–1 B (B–1) cell depletion. This finding is consistent with the observations that IFNγ inhibits proliferation of unsorted peritoneal cells but not purified B–1 or splenic B cells in response to LPS (A. O'Garra, M. Howard, unpublished), and that it inhibits the CFU response of normal peritoneal B cells stimulated with IL–5 and mitogens (107). The ability of IL–10 to downregulate

macrophage-dependent production of IFNγ by Th cells and NK cells may thus account for the striking effects seen when IL–10 is neutralized in vivo.

In addition to depletion of Ly–1 B (B–1) cells, anti-IL–10 treatment also led to an increase in serum levels of IgG_{2a} and IgG_{2b} antibodies, probably as a result of elevated levels of IFNγ, and possibly of IL–6. Furthermore, anti-IL–10 treated mice had elevated levels of circulating TNFα and were much more susceptible to death by LPS-induced septic shock, a monokine-mediated inflammatory reaction (108).

IL–10Δ mice have been produced by homologous recombination in embryonic stem cells (R. Kuhn, K. Rajewsky, W. Müller, personal communication). Preliminary observations showed that, in contrast to anti-IL–10–treated mice, IL–10Δ mice do not lack Ly–1 B (B–1) cells, nor do they have detectably elevated levels of circulating IFNγ, TNFα, or IL–6; furthermore, their circulating Ig levels appear normal. Conceivably, absence of endogenous IL–10 throughout development (although it is possible that maternal IL–10 could exert an effect) allows compensating regulatory mechanisms to emerge. Alternatively, the effects seen in anti-IL–10 treatment may be due in part to immune responses against xenogeneic anti-IL–10 antibodies or their immune complexes, which in the absence of IL–10 could lead to enhanced IFNγ levels and consequent effects on the Ly–1 B (B–1) cell compartment. Further studies of the two systems may clarify the reasons for the contrasting observations.

CONCLUDING REMARKS

In the three years since its discovery, understanding of the biology of IL–10 has grown very rapidly, and substantial further progress is anticipated. It is now clear that IL–10 plays a major role in the regulation of immune responses as an inhibitor of macrophage and T cell effector function. A possible role in controlling development of the class of an immune response has also been suggested. Further research will also lead to better understanding of the significance of the pleiotropic activities of IL–10, and of the importance of vIL–10 in the interaction of EBV with the host immune system.

The known functions of IL–10 suggest possible clinical applications. IL–10's inhibition of macrophage activation and T cell cytokine synthesis suggest use as an antigen-nonspecific suppressor factor in certain T cell–mediated autoimmune diseases, or as an anti-inflammatory agent. The selective suppression of T_h1-like immunity by IL–10 in vitro also suggests possible use, either alone or in combination with other cytokines such as IL–4, in averting allograft rejection, especially since expression of T_h2 cytokines has been associated with experimentally induced allograft tol-

erance in certain animal models (109). Enhancement of T_h2 immunity by IL–10 and/or IL–4 suggests their possible use as adjuvants for vaccines where enhanced antibody production is desired. Similarly, antagonists of IL–10 may selectively enhance T_h1 immunity, which would be of possible benefit in infectious diseases of viral origin or in those involving bacteria or parasites that are intracellular pathogens. In addition, production of IL–10 by some types of tumors (31) may prevent an adequate immune response against the transformed cells, and IL–10 antagonists may prove useful in such situations.

That IL–10 inhibits IL–2-induced cytokine synthesis but not LAK activity suggests its possible use in combination with IL–2 in LAK therapy, as a means of inhibiting side effects of this treatment which may be cytokine-mediated (110, 111). Future experiments using various animal models of disease and autoimmunity should indicate the feasibility of these approaches.

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THE ROLE OF THE CD28 RECEPTOR DURING T CELL RESPONSES TO ANTIGEN

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KEY WORDS: costimulation, B7, immunosuppression, T cell activation, antigen presenting cell, tyrosine kinase, anergy

Abstract

The CD28 receptor is stimulated during the contact of T cells with antigen-presenting cells. A counter-receptor for CD28 is the B7 molecule expressed on activated B cells, dendritic cells, and macrophages. B7 also binds to CTLA–4, a receptor that is structurally related to CD28. CTLA–4 is expressed in low copy number by T cells only after activation, but it binds B7 with ~20-fold higher affinity than CD28. Inhibition of B7-CD28 interactions blocks immune responses in vitro and in vivo. Therefore, CD28 receptor stimulation is required for T cell responses to antigens and for B cell responses to T-dependent antigens. During T cell responses to antigens, CD28 receptor stimulation may be required to prevent clonal inactivation or anergy. CD28 receptor ligation induces tyrosine phosphorylation of specific substrates, including phospholipase $C\gamma_1$, and triggers both calcium-dependent and calcium-independent signals. The CD28 costimulatory receptor represents a novel target for immunosuppressive drugs.

INTRODUCTION

T lymphocytes require for activation distinct signals from the antigen presenting cell (APC) (1, 2). An antigen-specific signal is delivered through

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the antigen receptor following its engagement with antigenic peptides presented in the context of major histocompatability complex (MHC) molecules. However, it has been known for some time that antigen alone is insufficient to trigger an optimal immune reaction. Lafferty and coworkers showed that tissue cells of nonhemapoetic origin are not readily rejected in transplantation experiments (3). These findings were interpreted to mean that lymphocyte activation necessary for graft rejection requires second or costimulatory signals provided by APC. The requirement for costimulatory signals during lymphocyte activation has also been elegantly demonstrated in in vitro systems (4, 5). Although the molecular nature of these signals is not completely understood, several receptor/counter receptor pairs are now known that mediate adhesion and signal transduction between the T cell and APC (6). In this article, we review recent work on the T cell molecule CD28 which is a receptor for costimulatory signals provided by the B7 molecule on APC. We focus on progress made in two areas since the identification of B7 as a counter-receptor for CD28: (i) the role of CD28 in delivering T cell costimulatory signals in vitro and in vivo, and (ii) elucidation of CD28 signalling pathways.

Identification of CD28 as a Functional Receptor

CD28 was first identified in studies with anti-T lymphocyte mAbs as a differentiation antigen expressed by $CD4^+$ and some $CD8^+$ T cells (6a, 7, 8). The $CD28^-$ T cells were later identified as positive for the CD11b integrin receptor and were found to contain alloantigen-specific suppressive cells (8–10). CD28 is expressed at a detectable but low level on $CD4^+CD8^+$ thymocytes, and expression is increased by activation in vitro (11). Mature $CD3^+$ thymocytes have higher levels of CD28 and are similar to peripheral T cells in CD28 expression. CD28 was recently identified by specific mAb in the mouse and found to be expressed at a high level on immature thymocytes (12, 13). Human and mouse CD28 receptors therefore differ in expression levels at early stages of T cell differentiation. CD28 has also been found on plasma cells in man (14), but it is not yet clear whether CD28 is functionally active on plasma cells.

CD28 was also recognized in early studies with mAbs as an important receptor regulating T cell activation. T cell proliferative responses were dramatically enhanced by including anti-CD28 mAb 9.3 together with polyclonal activators such as PMA, PHA, anti-CD3, or anti-CD2 mAbs (15–21). In addition to augmentation of proliferation, anti-CD28 mAbs together with anti-CD3 mAb increase T cell cytolytic activity (22, 23). These functional CD28 studies were recently reviewed (24). In these assay systems, anti-CD28 mAbs were found to increase expression of IL–2R α chain (CD25) and coordinately to increase production of Th1 cytokines

including IL–2, IFNγ, GMCSF, and TNFα (25–27). More recent studies have shown increased synthesis of IL–1, IL–3, and IL–4 following T cell activation with anti-CD28 mAbs (20, 28–30). Lindsten et al (31) showed that the effects of anti-CD28 mAb on Th1 cytokine production resulted from coordinate stabilization of the mRNAs for these cytokines. Anti-CD28 mAb also regulate transcription of the IL–2 gene (32–35). Together, these studies support the view that CD28 naturally functions as a major regulator of T cell cytokine gene expression. CD28 may also play a role in the pathogenesis of HIV, as suggested by studies showing activation of the HIV LTR by anti-CD28 mAbs in cell culture models (36, 37).

Structure of the CD28 Family of Cell Surface Receptors

Cell surface labeling in immunoprecipitation techniques established that CD28 is a homodimeric molecule, comprising $M_r = 44{,}000$ subunits (38). A cDNA for CD28 was cloned by Aruffo & Seed (38) using a novel expression cloning procedure. The predicted sequence of CD28 is that of a receptor of the immunoglobulin supergene family (39), with a single V_H-like domain in the extracellular region, a transmembrane domain, and a short intracellular domain. The gene structure of CD28 was established by Lee et al (40). These investigators also identified an alternatively spliced form of CD28 mRNA that encodes a truncated molecule lacking much of the V_H-like domain. Murine (12) and rat (41) homologues of CD28 have been cloned and shown to have ~67–68% sequence identity with human CD28.

CD28 bears structural homology to CTLA–4, a predicted T cell surface molecule identified by differential screening of a murine cytolytic-T cell cDNA library (42, 43). CTLA–4 transcripts were found in T cell populations having cytotoxic activity, leading to the suggestion that CTLA–4 might function in the cytolytic response (42, 43). Human CTLA–4 bears ~76% overall identity to its murine counterpart, but the cytoplasmic domains of these molecules are completely conserved across species (44). The overall level of sequence homology between CTLA–4 and CD28 in either human or mouse is ~20%, with 5 conserved cysteine residues. The region of greatest homology between CD28 and CTLA–4 is adjacent to the carboxy terminal Ig-like cysteine and contains the unusual hexapeptide, MYPPPY, which is completely conserved in CD28 and CTLA–4 from all species. Genes for CD28 and CTLA–4 share the same intron/exon organization (45) and colocalize to the same chromosomal regions in human (region 2q33–34) and mouse (45–47). These genes have also been linked at the molecular level, because both are found on individual clones from a yeast artificial chromosome library (48). Thus, CD28 and CTLA–4 are genetically linked and are evolutionarily related proteins.

IDENTIFICATION OF A COUNTER RECEPTOR FOR CD28 AND CTLA–4

In contrast to their potent stimulatory effects on proliferation induced by polyclonal stimuli, anti-CD28 mAbs inhibited antigen-specific proliferation in vitro. Damle et al (49) first showed that anti-CD28 mAb 9.3 blocked autologous mixed lymphocyte reactions (MLR). A subsequent investigation of these effects (50) showed that Fab fragments of mAb 9.3 were better inhibitors of MLR than the intact mAb, and that the inhibitory effects of mAbs were reversed when anti-CD28 mAbs were cross-linked on the cell surface with anti-mouse kappa mAbs. Lesslauer et al (51) showed that mAb 9.3 inhibited activation of class II–restricted T cell clones reactive with *M. leprae* antigens.

The inhibitory effects of anti-CD28 mAbs suggested that CD28 had an essential function during T cell interactions with APC. One possibility was that CD28 mediated adhesion between T cells and the APC. The structure of CD28 (38) was that of a member of the immunoglobulin (Ig) superfamily of molecules, many of which function as cell adhesion molecules (39). These findings prompted a search for a natural ligand for CD28 on APC. We developed an assay for intracellular adhesion mediated by CD28 (52). CD28 was transfected into Chinese hamster ovary (CHO) cells, and a cell line was isolated that expressed high levels of CD28 on the cell surface. This cell line mediated specific intercellular adhesion with human lymphoblastoid and leukemic B cell lines, and with activated primary murine B cells. CD28–mediated adhesion was not dependent upon divalent cations, so adhesion assays were routinely performed in the presence of EDTA to minimize intracellular adhesion mediated by other cation-requiring molecules. CD28 mediated–adhesion was blocked by anti-CD28 mAb 9.3, and by several mAbs reactive with lymphoblastoid cells, including mAbs against MHC class I antigens, and the BB–1 MAb against the B cell activation antigen, B7/BB–1 (53) (hereafter referred to as B7). The significance of CD28-adhesion inhibition by anti-MHC class I mAbs remains poorly understood, since class I expression by different B cell lines did not correlate with their ability to adhere to $CD28^+$ CHO cells. In contrast, expression of B7 antigen by B cell lines correlated well with their ability to adhere to $CD28^+$ CHO cells. Furthermore, transfection of COS cells with B7 cDNA (53) conferred the ability to adhere specifically to CD28; this adhesion was blocked by mAbs to CD28 and B7. These findings first demonstrated interaction between CD28 and the B7 molecule, which was further characterized using genetic fusions of the extracellular portions of B7 and CD28, and Ig $C\gamma_1$ chains (54). ^{125}I-labeled B7Ig bound to $CD28^+$ transfected CHO cells, and to immobilized CD28Ig with a Kd $\sim$200 nM.

B7Ig also inhibited adhesion of lymphoblastoid cells to $CD28^+$ CHO cells. Immunoprecipitation and binding inhibition studies showed that CD28 was the primary receptor for B7Ig on activated peripheral blood T cells. These studies clearly showed that B7 was a counter receptor for CD28.

CTLA–4 was identified as a second receptor for B7 (55). CTLA4Ig, a soluble Ig fusion of the extracellular region of CTLA–4, was expressed in COS cells (55). Like the analogous CD28Ig fusion protein (54), CTLA4Ig bound ^{125}I-B7Ig, but with ~20-fold higher avidity (Kd = ~12 nM). Thus, B7 is counter receptor for two T cell molecules, CD28 and CTLA–4, as represented in Figure 1. CTLA–4 is co-expressed with CD28 on activated T lymphocytes (56), but at much lower levels compared with CD28. The functional role of CTLA–4 during T cell activation remains to be defined. Several properties of CD28, CTLA–4 and B7 are summarized in Table 1.

Structure and Expression of the B7 Counter Receptor

The B7 antigen was first identified by Yokochi (57) as a B lymphoblast antigen recognized by mAb BB–1. Freedman (58) later produced mAb B7 which recognized a molecule present on activated B cells. Neither of these early studies demonstrated a function for B7, although Freedman (58) showed that $B7^+$ B cells more rapidly entered the cell cycle after cell activation than did $B7^-$ B cells. Freeman (53) determined the structure

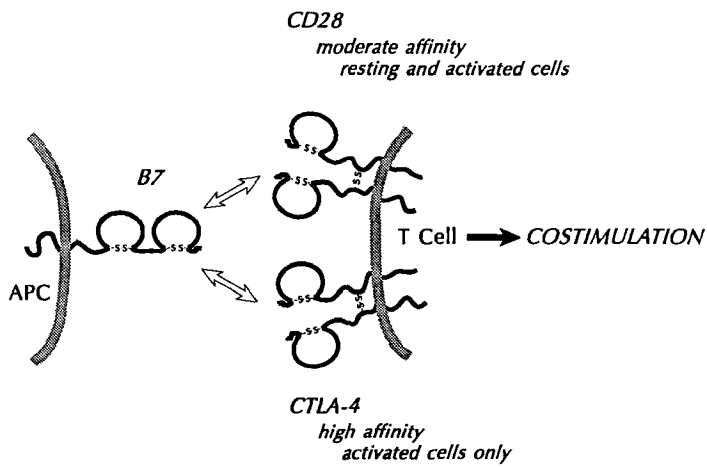

Figure 1 Lymphocyte interactions mediated by CD28, CTLA–4, and B7. Ig-like domains and subunit structure of these molecules are indicated. CTLA–4 and CD28 are homologous, but differ in their binding avidities.

Table 1 Expression of properties of CD28, CTLA-4 and their counter-receptors B7

	Expression	Function and signals
CD28	Most $CD4^+CD8^+$ thymocytes and peripheral $CD4^+$ or $CD8^+$ except $CD11b^+$ suppressor cells. Also found on plasma cells. Increased expression occurs on T cells and thymocytes after anti-CD3, PHA, or PMA, decreased by anti-CD28 activation. Binds to B7 directly with Kd = 200 nM.	CD28 binding is required for antigen-specific T cell responses by regulating cytokine production by transcriptional and posttranscriptional mechanisms. Transduces both calcium dependent and calcium independent (cyclosporin A resistant) signals that require tyrosine kinase activation. PTKs $p56^{lck}$ and $p59^{fyn}$ are activated rapidly by CD28 crosslinking. Other signals (PMA, anti-CD3, PHA) increase coupling of CD28 to PTK and calcium signals.
CTLA-4	Expressed on activated $CD4^+$ and $CD8^+$ T cells. Regulated by CD28 signals. Low abundance but high affinity binding to B7 (Kd = 12 nM).	CTLA-4 has unknown functions, but may contribute to co-stimulatory signals with CD28 during binding to B7. CTLA-4 expression may also function to increase adhesion avidity between T cells and APC. CTLA4Ig fusion protein binds B7 and inhibits T cell responses in vitro and in vivo.
B7	Expressed on activated B cells and macrophages but not resting B cells or monocytes. Dendritic cells are $B7^+$. Expressed by virally transformed cells such as EBV^+ B cells and HTLV-1^+ T cells.	B7-CD28 interactions are indirectly required for B cell responses by controlling helper T cell cytokine production. B7 human and mouse homologue are not similar in the cytoplasmic domain, and B7 has not been found to directly transduce signals in B cells.

of the B7 molecule by expression cloning. B7 was characterized by an extracellular region containing two Ig-like domains (one V region–like and one C region–like), a transmembrane domain and a short cytoplasmic tail. While B7 is clearly a member of the Ig superfamily, sequence homology between B7 and other family members was limited. B7-transfected COS cells bound both the B7 and BB–1 mAbs, indicating that these mAbs recognized the same molecule. More recently, Freeman (59) determined the structure of the murine homologue of B7 by cDNA cloning. Murine B7 had structural features similar to human B7, but the two molecules shared surprisingly limited sequence identity at the amino acid level. Alignment of the mature (without signal peptide) murine and human B7 molecules showed ~45% amino acid identity. In contrast, murine and human CD28 share ~67% identity, and murine and human CTLA–4, ~76% identity. Thus B7 apparently has greater sequence divergence between human and mouse than does either of its two receptors. It is also noteworthy that the cytoplasmic domains of murine and human B7 share very little homology, arguing against a signal transducing function for this region. The low degree of sequence homology between murine and human B7 may suggest the existence of a second B7-like molecule which perhaps has greater conservation between human and mouse. As with human B7, murine B7 binds to human CTLA4Ig more strongly than human CD28Ig (60).

Chromosomal mapping studies for the human B7 gene have given conflicting results. The BB–1 antigen was first mapped to chromosome 12 by staining of somatic cell hybrids between human leukemic cells and murine cells with mAb BB–1 (61). The B7 gene was then localized to human chromosomal region 3q13.3–3q21 by a combination of PCR analysis of a panel of hamster-human somatic cell hybrid DNAs and in situ hybridization of metaphase chromosomes (62). Since the latter study used more precise technology, it is tempting to believe that the earlier study was in error. Alternatively, results of the earlier study may indicate that a gene controlling B7 expression maps to chromosome 12, or that another cell surface molecule other than B7 maps to chromosome 12 and binds mAb BB–1.

B7 was first identified on activated B cells, and expression was shown to be upregulated during B cell activation (53, 58, 59, 63). More recent studies have shown B7 expression on other cell types. Freedman et al (64) showed that B7 was expressed on γ-interferon treated monocytes. Valle et al (65) described a new mAb, mAb 104, which reacts with B7, and showed that this molecule is expressed strongly on HTLV-I transformed T cells. Razi-Wolf et al (66) showed B7 expression on murine peritoneal exudate cells as detected with mAb. Finally, Liu et al (60) showed B7 expression

on murine dendritic cells by binding of CTLA4Ig. These studies indicate that expression of B7 is not limited to activated B cells as originally thought, but rather is more generally characteristic of APC.

Costimulatory Activity of the B7 Counter Receptor

Kohno et al (67) first suggested a receptor-like function for CD28 in mediating B cell–induced IL–2 production by T cell leukemia cell lines. The identification of B7 as a counter receptor for CD28 suggested a mechanism for T-B cell collaboration. Because of the extensive work showing T cell activation with anti-CD28 mAbs, it was easy to envision how the B7 counter receptor on activated B cells could activate or costimulate T cells via interaction with CD28. This was borne out when several studies soon thereafter demonstrated T cell costimulation by B7. Immobilized B7Ig fusion protein and $B7^+$ CHO cells augmented anti-CD3–induced T cell proliferation (54). $B7^+$ CHO cells also stimulated production of IL–2 mRNA in preactivated T cells (54). Similar results were reported by Gimmi et al (68). These authors found that $B7^+$ CHO cells augmented proliferation and IL–2 secretion by T cells suboptimally activated with PMA or anti-CD3. Damle (69) showed that costimulation of T cell proliferation by B7Ig fusion protein was more effective on primed, than on resting T cells. Augmentation of murine T cell proliferation by B7-transfected cells has also been reported (59, 60, 66). Azuma (70) reported that a B7-negative Burkitt's lymphoma cell line became a potent stimulator of MLR following its transfection with B7. These authors also showed that transfection of B7 into murine Fc receptor-bearing P815 cells caused these cells to become targets for anti-CD2 or anti-CD3 redirected cytotoxicity by small, resting human T cells. This observation suggested that B7/CD28 interaction may be important in the early stages of a cell-mediated immune response.

The observation that anti-CD28 mAbs inhibited antigen-specific T cell proliferation in vitro (49, 51) suggested an important role for CD28 in accessory cell–mediated T cell proliferation. After identification of B7 as a counter receptor for CD28, it was important to determine if anti-B7 reagents also blocked antigen-specific responses in vitro. Anti-B7 mAbs were soon shown to block alloantigen responses (71, 72). Damle et al (72) also showed that T helper cell–induced Ig secretion by B cells was blocked by anti-B7 mAb. CTLA4Ig was found to be a more potent inhibitor of human alloantigen responses than either anti-CD28 mAbs or anti-B7 mAbs (55). The greater potency of CTLA4Ig than mAb BB–1 was probably the result of the greater avidity of CTLA4Ig for B7 (55). CTLA4Ig also binds murine B7 and blocks murine accessory cell–dependent T cell proliferation (60). Razi-Wolf et al (66) described production of a mAb to

murine B7 which blocked T cell costimulation by accessory cells. Together, these results strongly argue the fundamental importance of B7 costimulation in T cell activation.

CD28 and T Cell Anergy or Unresponsiveness

Using in vitro models, Schwartz and colleagues have shown that antigen presentation in the absence of T cell costimulation led to functional inactivation or clonal anergy of T cells (4). The anergic state is characterized by failure to produce IL–2 and can be corrected by addition of exogenous IL–2 (4). T cell stimulation in the absence of costimulation may also lead to activation-induced T cell death (73). Thus, it appears that T cell costimulatory pathways determine whether TCR engagement results in immune activation or inactivation (5), and they may play a role in development of peripheral tolerance.

The molecular basis for T cell costimulation is not well understood, but because of its function in regulating lymphokine production, recent work has focused on a role for CD28 regulating the development of anergy. Jenkins et al (74) showed that anti-CD28 mAbs augmented proliferation of human T cells in the presence of APC that were tetanus toxoid-pulsed, but deficient in costimulation. Costimulation by autologous APC, like that by anti-CD28 mAbs, was partially resistant to inhibition by cyclosporin (74). Harding et al (75) showed that mAb triggering of CD28 blocked induction of anergy in murine $CD4^+$ T cell clones. If CD28/B7 interactions are important for blocking induction of anergy, then blocking B7 would be predicted to induce unresponsiveness. Initial results suggest that blocking of primary human MLR with CTLA4Ig induced alloantigen-specific unresponsiveness of $CD4^+$ T cells during subsequent stimulation (76). This state of unresponsiveness was reversed by anti-CD28 mAb or IL–2. A role for CD28 in preventing activation-induced cell death of $CD4^+$ T cells from HIV-infected patients was suggested by the studies of Groux (77). Although induction of anergy and induction of cell death are undoubtedly complex, current data suggest that CD28 triggering prevents these processes and maintains T cell responsiveness. A diagrammatic representation of the role of CD28 in maintaining T cell responsiveness is shown in Figure 2.

CD28 Signals

CD28 signals have often been thought of as a "second signal" in T cell responses, because of the ability of anti-CD28 mAbs to augment other activation pathways, leading to increased cytokine production and proliferation. In general, resting T cells do not proliferate in response to CD28 stimulation alone, but primed or previously activated T cells are often

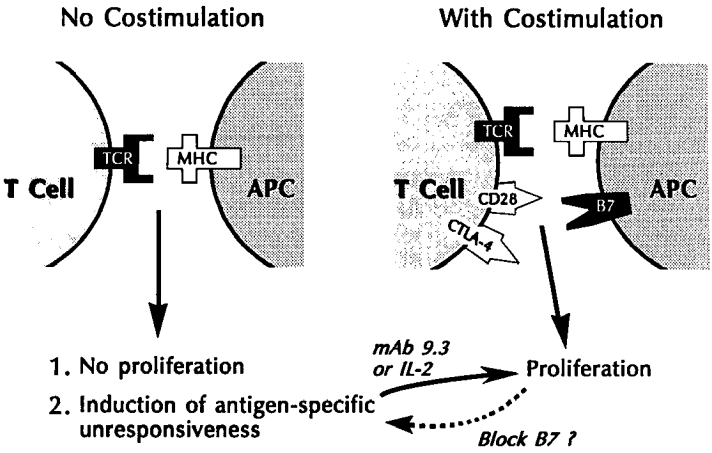

Figure 2 Role of CD28/CTLA-4/B7 interactions in determining T cell responsiveness to antigen. Shown is model for these interactions in controlling T cell commitment to an antigen-responsive or unresponsive state.

highly responsive to CD28 signals without requiring another receptor signal to induce IL–2 and subsequent proliferation. The ICAM–1/LFA–1 adhesion pathway seems to play an important role in regulating the responses to B7 stimulation by primed cells (78). CD28 cross-linking rapidly increases integrin-mediated adhesion in T cells (79). Therefore, the B7/CD28 and ICAM–1/LFA–1 adhesion/signalling pathways are interconnected. Liu & Janeway (80) have shown that costimulation of resting T cells by B7 is more efficient if B7 and TCR ligand are presented on the same rather than separated APC. This may indicate interaction between CD28 and components of the TCR complex, or that B7 signals must be temporally linked to TCR signals.

T cell responses through the CD28 receptor can also remain active for an extended period following the primary mitogen, or "first signal." For example, cells primed with PMA for 16 hr show heightened responsiveness to CD28 (27, 81, 82). Similarly, cells activated by PHA and expanded for six days without restimulation are highly responsive to CD28 triggering (83). Expression of CD28 is increased after anti-CD3, PMA, or PHA stimulation. These stimuli also increase coupling of CD28 to its signal transduction pathways, including the calcium signals (11, 27) (see below). Although CD28 signals may be effective when initiated long after the primary activation signal, CD28 signals are not effective when given first. Our evidence suggests that CD28 signals are desensitized by CD28 ligation

(84). This is associated with transient downregulation of CD28 expression and prolonged decreases in CD28-induced calcium signals. These examples show that both the expression of CD28 and signals coupled to the receptor are subject to positive and negative regulation.

The role of calcium in mediating CD28 signals has been extensively studied. When mAb 9.3 was used with PMA to drive proliferation, no increase in cytoplasmic calcium concentration $[Ca2^+]_i$ was apparent even when the cells were analyzed over several hours (82, 85, 86). Similarly, mAb 9.3 in solution was unable to increase the $[Ca2^+]_i$ response to anti-CD3 binding, even under conditions that were effective in augmentation of cytokine production (87). Binding of mAb 9.3 to CD28 therefore did not directly increase $[Ca2^+]_i$ and appeared to deliver a calcium-independent signal to T cells.

Further support for a calcium-independent arm of the CD28 signal comes from the observation that anti-CD28 mAb could lead to cyclosporin A (CsA)–resistant IL–2 gene expression and T cell proliferation (88). CsA forms a complex with cyclophilin that inhibits the activity of calcineurin (89, 90), a serine/threonine phosphatase that is calcium and calmodulin dependent. Calcineurin has now been identified as a functional target of CsA required for the induction of NF-kB and NF-IL2A transcription factors (91, 92). CsA therefore inhibits responses to calcium mobilization signals through its effect on the calcium-regulated enzyme calcineurin. The resistance of CD28 signals to inhibition by CsA extends also to the drug FK506 and has been observed in several labs (93–98). The CsA and FK506 resistant CD28 pathway therefore reflects the calcium-independent CD28 pathway signal during T cell activation.

Anti-CD28 mAbs are synergistic or interactive with combinations of PMA and ionomycin in their ability to stimulate IL–2 production by T cells (32, 86). Because PMA and ionomycin are pharmacological stimulants of protein kinase C (PKC) and calcium mobilization, respectively, these results further support the evidence for a calcium-independent CD28 signal pathway. A study of the PKC inhibitors H7 and staurosporine showed that CD28 responses were not PKC dependent, whereas CD3 responses were PKC dependent (99). Since PKC activation by phospholipase C (PLC) is associated with increases in $[Ca2^+]_i$, these results are consistent with a CD28 pathway that is calcium independent. Non-cross-linked anti-CD28 mAb is active in delivering these calcium-independent signals which are associated with the post-transcriptional effects on cytokine mRNA half-life (100) and on the transcriptional regulation of IL–2 mRNA expression (32–35).

Although the evidence convincingly points to a calcium-independent and CsA resistant CD28 signalling pathway, there is also direct evidence

that CD28 cross-linking on the cell surface with a second-step antiglobulin induces increased $[Ca2^+]_i$ and production of inositol trisphosphates in normal T cells (27, 101). The calcium signal requires a higher degree of CD28 aggregation and is associated with increased cell proliferation responses (101). Because CD28-induced calcium signals are inhibited by co-cross-linking with the tyrosine phosphatase CD45 (102), the possibility that CD28 signals are initiated by tyrosine phosphorylation has been studied. CD28 triggering by either mAb 9.3 or by $B7^+$CHO cells induces tyrosine phosphorylation of restricted substrates (81, 82), including $PLC\gamma_1$ (83). Thus, CD28 signalling involves protein tyrosine kinases (PTK). CD28-induced phosphorylation required mAb cross-linking. Tyrosine phosphorylation of $PLC\gamma_1$ is associated with activation of the enzyme, suggesting that $PLC\gamma_1$ activation is responsible for the inositol trisphosphate generation and the calcium signal. This is also supported from the observations that the protein tyrosine kinase inhibitor herbimycin A can prevent the calcium signals from CD28 receptor cross-linking and the tyrosine phosphorylation of $PLC\gamma_1$ (82, 83).

We conclude that CD28 binding by mAb 9.3 in solution delivers a calcium and CsA independent signal and that further CD28 cross-linking enhances the response and gives $[Ca2^+]_i$ and PI turnover via activation of $PLC\gamma_1$. This is consistent with the partial inhibition of CD28 responses by CsA seen in some studies (33, 103). Both the calcium independent and calcium dependent pathways seem to be initiated by PTK activity. A diagrammatic representation of CD28 signalling pathways is shown in Figure 3.

CD28 triggering confers resistance to suppression of proliferation by increased cAMP in T cells (87, 93, 104). Increasing intracellular cAMP levels, either with the soluble analogue dBcAMP or by stimulation of endogenous cAMP with PGE2 or forskolin, suppresses T cell proliferation by preventing IL–2 production (105–108). Costimulation with soluble anti-CD28 mAb prevented the suppression of proliferation, whereas costimulation with anti-CD5 mAb did not alter the cAMP suppression (87). In the study by Nunes *et al.* (104), the CD28 signals that confer resistance to cAMP were found to be distinct from the CD28-mediated calcium signals, providing additional evidence for two signal transduction pathways related to early and late responses via the CD28 receptor.

Since mAb binding to CD28 appears to give at least two kinds of signals, it is important to ask about signals delivered by CD28 engagement with the B7 ligand during T cell interaction with APC. Here, two sets of experiments suggest that the natural B7 ligand delivers both calcium dependent and calcium independent signals. The first evidence derives from studies where mAb 9.3 in solution was found to inhibit rather than enhance

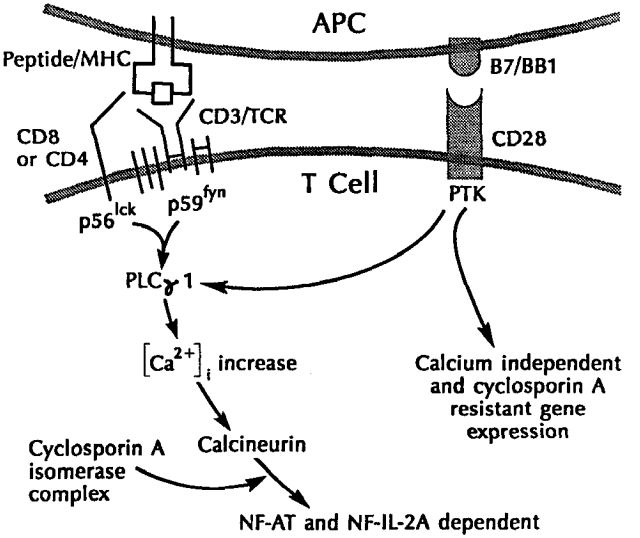

Figure 3 Signal transduction by the CD28 receptor. Protein tyrosine kinase (PTK) activation is an initial event that controls: (1) a calcium-dependent arm through the activation of phospholipase Cg1 (PLCg1); and (2) a calcium independent arm that confers resistance to cyclosporin A.

T cell responses in certain assay systems (49–51). These assays measured T cell responses to peptide antigen or to alloantigen, and they have in common the presence of B7 on the APC or the B lymphoblastoid cell line used for alloantigen presentation. These results show that binding of anti-CD28 mAb does not replace the B7 ligand and suggest that natural stimulation of CD28 in the system requires more than just the calcium-independent pathway triggered by mAb in solution. It is important to note that further CD28 cross-linking with mAb 9.3 plus a second step Ab did not block T cell responses to alloantigen but could directly stimulate the primed cells to secrete IL–2 and to proliferate (50). A direct investigation of the nature of signals delivered by B7 is shown in Table 2. T cell proliferation induced by $B7^+$ CHO cells is partially resistant to CsA, showing that the normal CD28 ligand triggers this calcium-independent signal.

In another direct examination, IL–2 mRNA expression by T cells stimulated with $B7^+$CHO cells or with mAb 9.3 and second-step antiglobulin was compared for their sensitivity to inhibition by CsA. Although the response to PMA plus ionomycin was inhibited >97% by CsA, the response to CD28 cross-linking was inhibited by only 30–60% (83). Both B7 and cross-linked 9.3 mAb induced the CsA resistant and the CsA

Table 2 CD28 stimulation directly induces proliferation of activated T cells by a cyclosporin A resistant pathway[a]

Stimulation[b]	Mean proliferation (^{3}H-thymidine incorporation, cmp $\times 10^{-3}$)[c]		
	− Cyclosporine	+ Cyclosporine (0.5 μg/ml)	
			% Inhibition
None	11.5	1.4	88
Immobilized anti-CD3	245.8	1.3	99
9.3 + 187.1	172.3	205.7	0
B7$^+$CHO	172.0	144.1	26
CD7$^+$CHO	11.6	1.2	91

[a] Activated T cells were generated from peripheral blood mononuclear cells with PHA-P (Wellcome) at 1 μg/ml followed by 6 days of culture in RPMI plus 10% fbs without additional PHA.

[b] T cells (5×10^4/well) were stimulated in microtiter plates as indicated using anti-CD3 mAb G19-4 immobilized on the plate, by anti-CD28 mAb 9.3 (1 μg/ml) followed after 5 min with anti-K mAb 187.1 (4 μg/ml), or by irradiated B7$^+$CHO or CD7$^+$CHO cells at 1:25 CHO:T cell ratios. In the absence of T cells, CHO cells alone gave < 1000 cpm.

[c] Proliferation was measured in quadruplicate cultures by uptake of 3H thymidine during the last 6 hours of a 3 day assay. Standard errors did not exceed 7% of the mean at any point.

sensitive component of the signal. Taken together, the data suggest that B7 uses both arms of the CD28 signal during cell-cell contact. In addition, because CD28 triggering with mAb 9.3 in solution inhibits T cell responses to antigen, both arms of the CD28 signal are required for T cell response to antigen.

Several labs have now found that CD28 cross-linking induces rapid tyrosine phosphorylation of proteins of approximately 96–100 kDa and 73 kDa in primed T cells (81–83). The tyrosine phosphorylation response is rapid and peaks at 2–3 min after CD28 cross-linking. The CD28-induced substrates appear to be a subset of those induced by CD2 or CD3 cross-linking, and they differ from the IL–2–induced substrates. We have also found that CD28 cross-linking induces tyrosine phosphorylation of PLCγ_1 and causes rapid production of inositol trisphosphates and calcium mobilization (83). We examined the p56^{lck}, p59^{fyn}, and p62^{yes} *src*-family protein tyrosine kinases in immunokinase assays. Both the p56^{lck} and p59^{fyn} but not p62^{yes} activities were consistently increased by CD28 cross-linking, and a rapid (within 30 min) shift in mobility of p56^{lck} to p60^{lck} was observed. Although these results may implicate p56^{lck} and p59^{fyn} in the CD28 signalling pathway, they do not necessarily indicate that these kinases initiate the signal. No tyrosine kinase activity has yet been found associated with CD28 in either NP40 or digitonin lysates (83). The p56^{lck} and p59^{fyn} kinases have already been implicated in CD4 and CD3/Ti signals and have been

found in direct association with the receptors. Further, $p56^{lck}$ is activated rapidly during IL–2–induced responses and also by CD2 receptor cross-linking. It is not yet been clear how the biologically distinct receptor signals could be transduced by $p56^{lck}$ and $p59^{fyn}$ in every case. Possibly another protein tyrosine kinase exists that is activated by bivalent CD28 binding to transmit calcium-independent, CsA resistant signals. Further CD28 cross-linking, as was done in our experiments, could recruit $p56^{lck}$ and $p59^{fyn}$ to amplify the response through a $PLC\gamma_1$ and calcium-dependent, CsA-sensitive pathway.

Function of CD28 In Vivo

This review emphasizes the importance of CD28 signalling in (i) regulating T cell proliferation, and (ii) maintaining T cell responsiveness in vitro (Figure 2). These results suggest that CD28 and B7 would represent new targets for immune intervention in vivo. CD28 stimulation could be useful when immune stimulation is desirable (i.e. in patients having infections or tumors). Alternatively, blocking CD28/B7 interactions or CD28 induced signals could be useful when immune suppression is desired, as in transplantation or autoimmunity.

The latter possibility has been tested in in vivo experiments. We have tested the effects of CTLA4Ig on in vivo immune responses to model immunogens (109). CTLA4Ig treatment potently blocked in vivo antibody responses to sheep erythrocytes and to keyhole limpet hemocyanin (KLH). Antibody responses to KLH were suppressed even if treatment with CTLA4Ig was delayed for up to two days following immunization. This suggests that B7 costimulation is most likely involved in maintaining or amplifying, rather than initiating, the response to this antigen. Animals immunized with sheep erythrocytes showed reduced in vitro T cell responses to antigen. Treatment with high doses of CTLA4Ig led to prolonged immunosupression, but not permanent "tolerance." These results demonstrate the importance of costimulation by B7 for humoral immune responses in vivo.

Lenschow (110) studied the effects of CTLA4Ig on xenogeneic islet cell transplantation. Mice were treated with streptozotocin to destroy pancreatic β cell function and were then transplanted with human pancreatic islets. CTLA4Ig treatment blocked islet cell rejection. mAb BB–1 which reacts with human B7, but not murine, also blocked rejection, indicating that human APC are involved in graft rejection. In contrast to the results with suppressing antibody responses (109), in this model CTLA4Ig treatment induced long-term donor-specific unresponsiveness. Differences in the T cell subsets, APC, or antigens involved may account for differences in the studies of Lenschow (110) and Linsley (109) to induce

"tolerance." These in vivo studies demonstrate that CD28/B7 interactions or signalling pathways represent targets for novel immunosuppressive drugs.

CONCLUSIONS AND FUTURE DIRECTIONS

The ligation of CD28 by B7 during T cell responses to antigen is required for IL–2 production and proliferation of T cells. CTLA4Ig is a potent inhibitor of B7 binding and is effective in vivo in blocking B cell responses to T helper dependent antigens. CTLA4Ig was also effective in preventing rejection of pancreatic islet xenografts and was able to induce long-term graft survival without additional CTLA4Ig. Inhibition of the B7-CD28 pathway may therefore lead to tolerance-induction in vivo for transplantation and the inhibition of antibody responses during autoimmunity.

CD28 signalling requires activation of PTK and involves a cyclosporin A–resistant, calcium-independent arm and also a calcium-dependent signal involving PLCγ_1. Progress in this area will rely on identification of the direct mediators of the novel CD28 calcium-independent signal and the discovery of specific inhibitors of these signals.

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NOTE ADDED IN PROOF: Two studies recently accepted for publication have bearing on topics discussed in this review. One study provides evidence that CTLA-4 is coexpressed with CD28 on activated T lymphocytes and cooperatively regulates T cell adhesion and activation by B7 (P. S. Linsley, J. L. Greene, P. Tan, J. Bradshaw, J. A. Ledbetter, C. Anasetti,

N. Damle. Coexpression and functional cooperativity of CTLA-4 and CD28 on activated T lymphocytes, *J. Exp. Med.*, in press, 1992). The second study shows that expression of B7 on murine tumor cells costimulates anti-tumor immunity mediated by $CD8^+$ T cells (L. Chen, S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, P. S. Linsley. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell*, in press, 1992).

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PEPTIDES NATURALLY PRESENTED BY MHC CLASS I MOLECULES

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Abstract

MHC class I molecules are peptide receptors of stringent specificity which however still allow millions of different ligands. This is achieved by the following specificity characteristics summarized as allele specific peptide motifs: Peptides are of defined length, depending on the class I allele (either 8 or 9 residues; exceptions have been observed). Typically, 2 of the 8 or 9 positions are anchors that can only be occupied by a single amino acid residue, or by residues with closely related side chains. Location and characteristics of anchors vary with class I alleles. The C terminus of the peptide ligands is frequently an aliphatic or charged residue. Such allele-specific class I peptide ligand motifs, known so far for H–2K^d, K^b, K^k, K^{km1}, D^b, HLA-A*0201, A*0205, and B*2705, are useful to predict natural T cell epitopes. The latter can be determined by extraction from cells recognized by the T cell of interest. It is not known how the class I ligands are produced in the cell, although speculative models exist. The peptide specificity of class I molecules and experimental evidence indicate that T cells are tolerant to only a small fraction of the expressed genomic sequences and are not tolerant to the remainder. The function of class I molecules is to present a collection of self-peptide samples at the cell surface for surveillance by T cells.

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INTRODUCTION

Major histocompatibility complex (MHC) molecules are peptide receptors. Cell surface MHC class I molecules present peptides derived from cellular proteins to T cells. In the normal situation, all peptides are from normal cellular proteins, and the T cells are tolerant to these normal self-peptides. In pathological situations, for example, if a cell has been infected by a virus, foreign peptides are presented in addition to the self-peptides. The foreign peptides can now be recognized by T cells, which may destroy these cells. Thus, the system of peptide presentation by MHC class I molecules and the screening of these peptides by T cells provide the immune system with a measure to survey the interior of cells for expression of abnormal proteins and to destroy such aberrant cells. This system is instrumental in protecting the body against viral disease, or against certain other infectious agents with cytosolic parasitism, like Listeria or Plasmodium. In addition, certain tumor cells expressing peptides unfamiliar to T cells may also be eliminated. The purpose of this review is to discuss the nature of peptides presented by MHC class I molecules, the rules for peptide presentation, the ways the peptides might be processed from cellular proteins, and the significance of peptide presentation for immunity as well as for self-tolerance.

STRUCTURE OF CLASS I MOLECULES

MHC class I molecules are glycoproteins consisting of a heavy chain, about 350 amino acids, and a light chain also known as β_2-microglobulin, about 100 amino acids (1). The human MHC is called HLA; that of the mouse H–2. Class I heavy chains as well as most other MHC genes are encoded on chromosome 6 in humans and on chromosome 17 of the mouse. The heavy chain consists of three extracellular domains, α_1, α_2 and α_3, a transmembrane region, and a cytoplasmatic tail. β_2-microglobulin is noncovalently attached to the α_3 domain. The α_1 and α_2 domains form a peptide binding groove made up of a β-pleated sheet and two alpha helices, as has been revealed by X-ray analysis of HLA-A2, Aw68, and B27 crystals (2–5). The extension of the peptide-accommodating groove is about 1×2.5 nm, and it holds peptides in roughly extended conformation of about 8 to 10 amino acids (5–7). The peptides are tightly bound in the groove; both N and C termini are buried and are interacting with MHC residues. In addition, certain side chains of peptide amino acid residues interact with corresponding MHC residues forming pockets, whose location and shape varies with the allelic forms of the molecule. The extreme polymorphism of MHC class I genes—both mice and humans

have a couple of dozen if not hundreds of alleles at each of the three class I loci, and one allele may differ from the other by 40 amino acids (1)—is reflected in different peptide-accommodating specificities of the respective grooves (2–7).

APPROACHES TO ANALYSIS OF CLASS I–ASSOCIATED PEPTIDES

The classical experiments showing that virus-specific T cells recognize viral antigen in the context of MHC class I molecules were those of Zinkernagel & Doherty (8). Such T cells could also be stimulated with synthetic peptides (9), and Townsend and colleagues localized these antigens on fragments of intracellular viral proteins, for which synthetic peptides bound to uninfected cells can also be substituted (10, 11). Isolation of naturally processed peptides recognized by class I–restricted T cells was not reported until 1990 (12). Two principle methods are used to isolate such peptides.

Acid Extraction of Peptides from Whole Cells

If cells are mechanically destroyed and treated with trifluoroacetic acid, the majority of material precipitates, whereas small peptides including class I ligands stay in the supernatant. The heterogeneous mixture of peptides and other molecules is then separated by gel filtration and HPLC, and the individual fractions are tested for recognition by T cells (12). This is done by incubating ^{51}Cr labeled target cells expressing the relevant MHC molecule, but not the relevant antigen, with the individual HPLC fractions. This process is followed by incubation with cytotoxic T cells specific for an antigen expressed by the cells used to prepare the extract. Such an approach is useful for isolation and characterization of peptides for which specific T cells are on hand. The first naturally processed T cell epitopes have been isolated this way; some of these were identified by biochemical comparison with synthetic peptides (12–14). An advantage of this approach is that it detects not only peptides that had been bound to MHC molecules in the intact cells, but also all other peptides that have the capacity to bind to MHC molecules and are recognized by T cells (15, 16).

Acid Extraction of Peptides from Purified MHC Molecules

Isolation of MHC class II molecules by immunoprecipitation followed by acid extraction of associated peptides has been reported by Demotz and colleagues (17). The first report on peptides isolated from purified class I molecules was by Van Bleek & Nathenson (18). The approach is to lyse cells with detergent, to precipitate MHC molecules by antibodies bound to a solid phase, and to dissociate peptides from MHC molecules by acid

treatment. Dissociated peptides are then separated by HPLC and can be analyzed by T cells, as with the previous approach. This led to identification of a natural vesicular stomatitis virus (VSV) epitope (18). Such MHC-eluted peptides are more readily accessible for sequencing due to less contamination with material not associated with MHC molecules. Partial sequence information of a class I–associated viral epitope has been derived by biosynthetic labeling of VSV-infected cells with radioactive amino acids Tyr and Leu; thus, the position of these residues but not of other residues was determined by this sequencing approach, confirming the identity of the VSV epitope (18). Direct sequencing of peptides, by Edman degradation and/or by mass spectrometry, allowed determination of complete ligand sequences of unknown protein origin (19–21).

THE ANTIGENIC SYSTEMS USED TO CHARACTERIZE NATURAL PEPTIDES RECOGNIZED BY CLASS I–RESTRICTED T CELLS

Peptides Derived from Foreign Proteins Expressed from Transfected Genes

Mouse cells expressing foreign proteins from transfected genes, like HLA, β-galactosidase, or ovalbumin, readily induce class I–restricted CTL in syngeneic mice and are recognized by such T cells (e.g. 22–25). The peptides recognized can be narrowed down using synthetic peptides or fragmented proteins, similarly as for virus-specific CTL. The natural peptide from β-galactosidase, as recognized by L^d-restricted CTL, has been isolated from β-galactosidase-transfected tumor cells (12).

Similarly, the natural peptide recognized by K^b-restricted CTL specific for ovalbumin was isolated, this time using purified K^b-molecules from transfected cells as source of peptide (26).

Minor Histocompatibility Antigens

Grafts exchanged between MHC-matched individuals can still be rejected, if other genes are different. Such non-MHC gene products leading to graft rejection are called minor histocompatibility antigens (27). Numerous genes of the sort are scattered throughout the mouse genome, many of which have been mapped and numbered: H–1, H–3, H–4, and so on. These antigens are recognized by MHC-restricted T cells (28). Minor H genes are also known in humans (29). The general idea is that such antigens are peptides derived from normal but polymorphic cellular proteins presented by MHC molecules, as first proposed by Townsend (10, 30, 31). Only few minor H genes have been identified so far; almost all are rather exceptional.

One is β_2-microglobulin whose allelic forms can be recognized by CTL (32–34), probably by conformational determinants, and not by recognition of β_2-microglobulin-derived peptide sitting in the MHC groove. Others are different allelic forms of a mitochondrially encoded gene (ND1, a subunit of NADH-dehydrogenase), peptides of which are presented by an odd class I molecule, Hmt, that is encoded telomeric of the H–2 region (35). (In addition, retroviral gene products can behave as minor H antigens—35a). Of those classical minor H gene products encoded autosomally and presented as peptide by classical MHC molecules, only one has been identified so far—the myxovirus resistance protein Mx recognized by K^k-restricted T cells (36). However, an additional one has been isolated as a protein (37), and several others as natural MHC class I ligands, like the K^b-restricted H–4^b antigen, the Y-chromosome encoded H-Y antigen (D^b-restricted) as well as an HLA-B35-restricted human minor H antigen (12, 15, 38).

Peptides Recognized by Alloreactive CTL

The trait leading to discovery of MHC genes was the strong rejection of MHC incompatible grafts in mice (caused by T cells) (1, 39). This reaction has its in vitro correlate in the strong activation of T cells confronted with stimulator cells expressing MHC. The specificity of such "alloreactive" T cells is rather heterogeneous. Some of such T cells might be peptide-independent, whereas a portion is clearly peptide-specific or at least peptide-dependent (16, 40–51). The analysis of naturally processed peptides recognized by several alloreactive CTL lines nominally directed against the H–$2K^b$-molecule has provided some insight into general aspects of protein processing in the class I pathway (16), a point discussed later. In addition, these experiments explained the high frequency of alloreactive T cells against any given foreign MHC molecule by the high number of combinatorial foreign MHC/foreign peptide complexes on any given foreign cell (16, 52, 53). The term "foreign peptide" in this context deserves closer consideration, a point discussed in the paragraph on self-tolerance.

Viral Peptides

The first naturally processed T cell epitopes to be identified were of viral origin. Identification was possible because the antigen recognized by the respective CTL had been narrowed down by transfection of individual viral genes, or truncated genes, and by analysis of CTL using synthetic peptides according to viral sequences, as pioneered by Townsend and colleagues (10, 11). The D^b-restricted influenza nucleoprotein nonapeptide ASNENMETM (Nucleoprotein 366–374) as well as the K^d-restricted nonapeptide from the same protein, TYQRTRALV (NP 147–156), were identi-

fied as natural T cell epitopes (13, 14) by comparison with longer synthetic peptide preparations known to contain the respective epitopes (11, 54). At the same time, van Bleek and Nathenson identified a K^b-restricted octapeptide of VSV nucleocapsid protein, RQYVYQGL, by eluting the natural peptide from purified K^b molecules (18). Only one additional natural viral epitope, the L^d-restricted nonapeptide YPHFMPTNL of mouse cytomegalovirus (MCMV) (55), has been identified to date (June 1992), although for many other cases likely candidates are known.

An important subsidiary point of the abovementioned work on influenza peptides was that the biological effects of a given synthetic peptide preparation may be due mainly to byproducts occurring in minute amounts. Crude synthetic IASNENMETMESSTLE (influenza nucleoprotein 365–380), for example, contained a very small amount of ASNENMETM that did not give an OD 220 signal in the HPLC profile and still was the most active fraction recognized by D^b-restricted, influenza-specific CTL (13, 14). This is due to the extremely high biological activity of natural epitopes, detectable in CTL assays down to the femtomolar range. This point was later confirmed with different peptide preparations and assays (56).

Peptides Recognized by Tumor-Specific CTL

Certain tumors in the mouse as well as in humans can be efficiently recognized by tumor-specific or, at least, tumor-directed CTL. Several genes coding for antigens recognized in such situations have been identified by the pioneering work of T. Boon and colleagues, for example, the antigens recognized on P815 mastocytoma cells of DBA/2, or of variants of these cells (57–60). Similarly, the antigen recognized by human melanoma-specific T cells has been identified (61). Although in these cases the genes as well as the approximate location of the T cell epitopes is known, the natural MHC ligand recognized by tumor-specific CTL was identified in only one case so far: The K^d restricted nonapeptide KYQAVTTTL is recognized by CTL specific for an immunogenic variant of P815 tumor cells (62).

THE RULES FOR PEPTIDES PRESENTED BY CLASS I MOLECULES

Pool Sequencing of Peptide Gemischs Eluted from Class I Molecules

The natural K^d-ligand TYQRTRALV (derived from influenza nucleoprotein), mentioned above, contains a Tyr (Y) residue, similar to several synthetic peptides known to contain K^d-restricted T cell epitopes and known to bind to K^d. Tyr is important for K^d-binding, as well as a Leu or

Ala residue 8 positions apart, as shown by J. Maryanski and coworkers (63). Alignment of the natural nonamer with those synthetic peptide sequences according to their Tyr residues suggested that all natural K^d-ligands might be nonamers with Tyr at position 2, and an aliphatic residue like Ile, Leu, or Val, at the C-terminus (14). This hypothesis was essentially confirmed when the natural K^d-ligands isolated from P815 tumor cells were sequenced as a pool (19; Table 1). Similar approaches were also useful for other class I molecules, like K^b, D^b, and HLA-A2 (19). The results indicated that each MHC class I allelic product has its own peptide specificity, a peptide motif, characterized by an allele-specific length of 9 (K^b, D^b, A2), or 8 (K^b) amino acid residues. Certain positions, like position 2 of K^d-ligands, are occupied by residues with similar side chains; such positions are called anchors. Positions frequently but not always occupied by similar residues are called auxiliary anchors. The characteristics of the ligands of a given MHC allelic product are summarized as peptide motifs (19). The peptide motifs known so far (mostly determined by pool sequencing) are those of H–$2K^d$, K^b, D^b, K^k, K^{kml}, HLA-A*0201, A*0205, and B*2705 and are compiled in Tables 1 through 6 .

Comparing these peptide motifs with synthetic peptides containing T cell epitopes suggested that most synthetic peptide epitopes determined so far were longer than the respective natural ligand. In addition, such comparison suggested in some cases that the allele-specific peptide length is not so much determined by the number of amino acid residues, e.g. 9 for D^b-ligands, but rather by the spatial length of peptides as mounted in the MHC groove, since, especially for some Pro- and Gly-containing T cell epitopes, the aliphatic C-terminus could not be aligned at position 9 but rather at position 10 or 11 (Example: SGPSNTPPEI of Adenovirus) (19). It was suggested that certain residues like Pro (inducing kinks in peptide stretches) or combinations of Pro and Gly might contract the spatial lengths of peptides (19). Although natural class I ligands with more amino acids than the nominal allele-specific residue number have not been determined yet, optimal binding as well as T cell recognition of a Pro-containing D^b-restricted epitope with 10 instead of 9 residues (64) and of a K^b-restricted peptide with 9 instead of 8 residues (55a, 56, 65) suggested that the above assumption was correct. The notion was indeed proven recently by analysis of monopeptidic class I crystals (65a,b).

Sequencing of Individual Peptides Eluted from Purified MHC Molecules

Information on the ligand specificity of individual MHC molecules can also be derived by comparing a set of individual peptides eluted from the

Table 1 K^d-motif (Ref. 19)

	Position[a]								
	1	**2**	3	4	5	6	7	8	**9**
Anchor residues		**Y**							**L**
									I
Frequent residues			N	P	M	K	T		
			I			F	N		
			L						
Also observed	K	F	A	A	V	H	P	H	V
	A		H	E	N	I	H	E	
	R		V	S	D	M	D	K	
	S		R	D	I	Y	E	V	
	V		S	H	L	V	Q	F	
	T		F	N	S	R	S	R	
	G		E	R	T	L	A	L	
			Q		G	T		T	
			K		E	N		Y	
			M						
			T						

										Source	Ref.
Natural ligands	T	Y	Q	R	T	R	A	L	V	Influenza nucleoprotein	13, 54
	S	Y	F	P	E	I	T	H	I	Protein tyrosin kinase JAK1	19, 66
	K	Y	Q	A	V	T	T	T	L	Tumor antigen of P815	102
	G	Y	K	D	G	N	E	Y	I	Listeriolysin (L. monocytogenes)	73
Likely ligands[b]	R	Y	L	E	N	G	K	E	T	L	HLA-A24	19, 123
	R	Y	L	K	N	G	K	E	T	L	HLA-Cw3	19, 22
	S	Y	I	P	S	A	E	K	I		Plasmodium circumsporozoite protein	19, 80, 124
	S	Y	V	P	S	A	E	Q	I		Plasmodium circumsporozoite protein	19, 80, 125

[a] Anchor positions in bold.
[b] Sequences that (i) fit to the motif and (ii) are contained in synthetic peptides recognized by the respective T cells, or correspond to the synthetic peptide with highest biological activity.

grooves. This approach has the advantage that the protein of origin can be tracked down. The first natural MHC ligand to be directly sequenced was SYFPEITHI (19), a prominent self-peptide occupying about 5% of K^d molecules on P815 tumor cells. A matching peptide stretch, SFFPEITHI,

Table 2 D^b motif (Ref. 19)

	Position								
	1	2	3	4	**5**	6	7	8	**9**
Anchor residues					**N**				**M**
Frequent residues	M	I	K		L				I
		L	E		F				
		P	Q						
		V	V						
Also observed	A	A	G	D		A	D	F	L
	N	Q	N	T		Y	E	H	
	I	D				T	Q	K	
	F	S				V	V	S	
	P					M	T	Y	
	S					E	Y	T	
	T					Q			
	V					H			
						I			
						K			
						P			
						S			

		Source	Ref.
Natural ligand	A S N E N M E T M	Influenza nucleoprotein	11, 13
Likely ligands	S G P S N T P P E I	Adenovirus	19, 64
	S G V E N P G G Y C L	LCMV	19, 126
	S A I N N Y A Q K L	SV 40	127
	C K G V N E E Y L	SV 40	127
	Q G I N N L D N L	SV 40	127
	F Q P Q N G Q F I	LCMV	128
	A S N E N M D A M	Flu NP 1968	79

was found in a human sequence, protein tyrosine kinase JAK 1 (66). Thus, the natural K^d ligand suggested that the corresponding mouse JAK 1 sequence was SYFPEITHI, a notion confirmed in the meantime by sequencing of the mouse gene (A. G. Harpur, A. Ziemiecki,

Table 3 K^b-motif (Ref. 19)

	Position[a]							
	1	2	3	4	5	6	7	8
Anchor residues					F Y			L
Frequent residues			Y					M
Also observed	R I L S A H	N G I	P I	R D E K T V N		T I E S Q P	N Q K G	I V

									Source	Ref.
Natural ligands	R	G	Y	V	Y	Q	G	L	VSV	18
	S	I	I	N	F	E	K	L	Chicken ovalbumin	26
	H	I	Y	E	F	P	Q	L	Self protein of P815	134
Likely ligands	F	A	P	G	N	Y	P	A	L	Sendai virus	56
	S	I	I	E	F	A	R	L	HSV-I glycoprotein B	K. Rosenthal, pers. communication

[a] Auxiliary anchor position underlined.

A. F. Wilks, K. Falk, O. Rötzschke, H.-G. Rammensee, unpublished observation).

The approach of directly sequencing individual MHC ligands has been followed up by several laboratories for both class II (67–69) and class I (20, 21, 70). The peptide motif of HLA-B*2705 has been determined this way by analyzing 11 complete or partial sequences of normal B27 ligands (20). The technique was greatly improved by using mass spectrometry for microsequencing, yielding complete or partial sequences of some 9 HLA-A2 ligands (21), that entirely confirmed the A2 motif as determined by gemisch sequencing. The natural ligands of the different class I allelic

Table 4a K^k-motif[a]

	Position							
	1	**2**	3	4	5	6	7	**8**[b]
Anchor residues		**E**						**I**
Frequent residues			K					
			N					
			Y					
			M					
Also observed	V		Q	L	A	N	T	
	F		I		G	K		
			L		P	H		
			F		T			
			P		V			
			H		F			
			T		S			

										Source	Ref.
Likely ligands	F	E	S	T	G	N	L	I		Influenza Haemagglutinin 2	129
	F	E	A	N	G	N	L	I		Influenza Haemagglutinin 1	129
	I	E	G	G	W	T	G	M	I	Influenza Haemagglutinin 2	129
	S	E	F	L	L	E	K	R	I	SV 40 T antigen	129
	Y	E	N	D	I	E	K	K	I	Plasmodium circumsporozoite protein	129
	S	D	Y	E	G	R	L	I		Influenza Nucleoprotein	129
	D	E	L	D	Y	E	N	D	I	Plasmodium circumsporozoite protein	129

[a] M. Norda, K. Falk, O. Rötzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee, submitted.
[b] The C-terminal residue appears to be invariantly Ile; the C-terminus can be at 8 as well as at 9 although the majority of natural ligands are probably octamers.

products—detected by direct sequencing or by comparison with synthetic peptides—are also compiled in tables 1 through 6 as well as some likely natural ligands. In addition, two naturally processed ligands have been described for L^d-molecules, YPHFMPTNL and LSPFPFDL (51, 55). Together with partial information on the L^d-specificity from a pool-sequencing attempt (19), and with information on optimal synthetic T cell epitopes (59, 71) the L^d motif is likely to be XPXXXXXL, with other aliphatic residues allowed at 9, and Ser tolerated at 2.

Table 4b K^{bm1}-motif[a]

	Position							
	1	2	3	4	5	6	7	8
Anchor residues								I
Frequent residues		E	K					
Also observed		Q	N	P	A			R
		G	Q		R			Y
		P	G		K			
			M					
			P					
			Y					

[a] M. Norda, K. Falk, O. Rötzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee, submitted.

A method slightly at variance to direct sequencing of MHC ligands is to label cells metabolically with individual radioactive amino acids before peptide elution and to determine the relative position of that residue within the ligand. This approach has been used for identification of a natural VSV epitope (18), to confirm frequent usage of Tyr at positions 3 and 5 and Leu at 8 of the K^b-motif and to determine the position of these residues in several natural ligands of K^{bm1} and K^{bm8} molecules (72).

Prediction of Natural T Cell Epitopes Using the Motifs

If the sequence of a cellular protein of immunological interest is known, comparing the sequence with the allele-specific peptide motif of a relevant MHC molecule indicates which stretches of the protein are likely to be natural MHC ligands. An example is chicken ovalbumin expressed in H-2^b mouse cells. The ovalbumin epitope recognized by K^b-restricted T cells had already been narrowed down to one contained in the sequence IINFEKL TEWTSSNVNEER (chicken ovalbumin 258–276) (24, 73). The K^b-restricted peptide motif requires an octamer with an aromatic residue at position 5 and an aliphatic one at 8 (19). Since the only aromatic residue in the above sequence is the Phe (F) at position 4, an additional residue at the N-terminus, Ser, was required by the motif, to result in the octamer SIINFEKL. This prediction was experimentally confirmed by elution of the natural ovalbumin peptide from K^b molecules, followed by biochemical comparison to synthetic SIINFEKL (26). Similarly, the K^d-restricted pep-

Table 5a HLA-A2.1-motif (A*0201) (Refs. 19, 21)

	Position										
	1	**2**	3	4	5	6	7	8	**9**		
Anchor residues		**L**							**V**		
Frequent residues		M		E K		V		K			
Also observed	I		A	G	I	I	A	E	L		
	L		Y	P	K	L	Y		I		
	F		F	D	Y	T	H	S			
	K		P	T	N	G	V	G			
	M		M	S	G	A		A			
	Y		S	V	F	P		H			
	V		R		V						
	S		D		H						
	G		V		P						
	T		L		A						
			N		R						
			W		D						
										Source	Ref.
Natural ligands[a]	S	X	P	S	G	G	X	G	V	-	69
		L	L	D	V	P	T	A	A	V	Human IP 30 signal peptide	69
	L	L	L	D	V	P	T	A	A	V Q A	Human IP 30 signal peptide	98
	L	L	L	D	V	P	T	A	A	V	Human IP 30 signal peptide	98
		G	X	V	P	F	X	V	S	V	-	69
		S	L	L	P	A	I	V	E	L	P 61, regulatory subunit of protein phosphatase 2A	69
		S	X	X	V	R	A	X	E	V	-	69
		K	X	N	E	P	V	X	X	X	-	69
		Y	L	L	P	A	I	V	H	I	-	69
		T	L	W	V	D	P	Y	E	V	-	69
											-	69
Likely ligands		I	L	K	E	P	V	H	G	V	HIV reverse transcriptase	19
		G	I	L	G	F	V	F	T	L	Influenza matrix protein	19,130, 133
		I	L	G	F	V	F	T	L	T V	Influenza matrix protein	19
		K	L	G	E	F	Y	N	Q	M	Influenza B NP	131

[a] X: Leu or Ile.

Table 5b HLA-A*0205-motif (Ref. 136)

	Position								
	1	2	3	4	5	6	7	8	9
Anchor residues									L
Frequent residues	V	Y	G	V	I			K	
	L	P	E	Y					
	I	F	D	L					
	Q	I		I					
Also observed	M		K		V	Q			
			N		T				
					L				
					A				
					R				

tide recognized by CTL specific for a variant of P815 tumor cells was predicted and demonstrated to be KYQAVTTTL (62). In the case of a Listeria protein, Listeriolysin, the epitope recognized by K^d-restricted CTL had not been narrrowed down before by synthetic peptides or truncated genes. The K^d-restricted peptide motif indicated 8 nonamers in the Listeriolysin sequence as candidates for K^d-ligands, one of which, GYKDGNEYI, was indeed the correct one (73a). Thus, the MHC allele specific peptide motifs are useful for identifying natural T cell epitopes in proteins of immunological interest.

Allele-specific Peptide Motifs Are Not Just "Binding Motifs"

The majority of peptides that have been described so far to bind to class I molecules do not adhere to the respective peptide motifs (e.g. 14, 63–65, 74–78a). This reflects the capacity of class I molecules to bind peptides of between 4 and 28 amino acids, no matter whether they do or do not contain the relevant motif. However, the binding of the natural ligands is in most cases several orders of magnitude better than binding of the "wrong" peptides (13, 14, 56, 79). Because the peptide motifs were determined not by screening for peptides that are the best binders but rather by analyzing the MHC ligands occurring naturally, these motifs incorporate each single step required for a peptide to become a natural MHC

Table 6 HLA-B27-motif (Ref. 20)

	Position								
	1	**2**	3	4	5	6	7	8	9
Anchor residues		**R**							
Also observed	R		Y	Q	K	S	T	E	K
	G		I	K	E	I	V	K	R
	K		V	L	P	V	G	V	Y
	A		W	D	G	A	I	L	F
			S	N	R	L	E	H	L
			I	E		P	R	Q	
			F	T				A	
			L	P					

									Source	Ref.
Natural ligands[a]	R	R	Y	Q	K	S	T	E	L	Human Histone H3, H3.3	20
	R	R	I	K	E	I	V	K	K	Human Hsp 89α	20
	R	R	V	K	E	V	V	K	k	Human Hsp 89β	20
	R	R	W	L	P	A	G	d	a	Human elongation factor 2	20
	R	R	S	K	E	I	T	V	R	Human RNA-dependent RNA-helicase	20
	G	R	I	D	K	P	I	L	K	Ribosomal protein	20
	f	R	Y	N	G	L	i	H	r	Rat 60 S ribosomal protein L28	20
	K	R	F	E	G	L	T	Q	R	-	20
	R	R	F	T	R	P	E	H	-	-	20
	R	R	I	S	G	V	D	R	Y	-	20
	A	R	L	F	G	I	R	A	K	-	20
Likely ligands	S	R	Y	W	A	I	R	A	K	Influenza nucleoprotein	132
	S	R	Y	W	A	I	R	T	R	Influenza nucleoprotein	20, 132
	K	R	W	I	I	L	G	L	N K	HIV gag p24	20, 135
	G	R	A	F	V	T	I	G	K	HIV gp 120	20

[a] Compiled from the single peptides shown below.
[b] Lower case letters: residues of lower confidence.

ligand. One of these steps, of course, is binding; others, however, include the processing of peptides that must be influenced by the specificities of the enzymes involved, and probably also the transport of either proteins

or peptides between cellular compartments (see below). It is, therefore, scientifically not exact, and sometimes rather misleading, to talk of allele specific peptide motifs as "binding motifs". In the case of K^d, on the other hand, a true peptide binding motif has been established by Maryanski and coworkers, and it has the same basic characteristics as the motif based on natural ligands (80). Thus, peptide binding motifs and natural ligand motifs of MHC molecules may coincide but should not be expected to do so a priori.

Peptide Specificity of "Nonclassical" Class I Molecules

In addition to the "classical" class I genes, HLA-A, B, C, and H–2K, D, L, many other class I genes are encoded on the respective chromosome; some of them are expressed on the cell surface (1). Some of those "nonclassical" class I molecules have been shown to present antigen in a few cases (e.g. 35, 81, 82). For the Hmt molecule, which presents a mitochondrially encoded minor H antigen, Mta, to CD8+ T cells (35), studies with synthetic peptides have indicated that this molecule is able to bind peptides formylated at the N-terminus (83). Although natural Hmt-ligands have not been identified yet, these data indicate that Hmt molecules are specialized in presenting formylated peptides that might occur as fragments of proteins from prokaryotes or mitochondria (83). Analysis of natural ligands of another nonconventional class I molecule, glycophosholipid-anchored Qa–2, indicated a peptide motif similar to that of ordinary class I molecules, except that it seemed to be much more stringent in its specificity. According to this study, Qa–2 ligands are nonapeptides with two anchors (His at 7 and L, I, or F at 9) and four auxiliary anchors (O. Rötschke, K. Falk, S. Stevanović, B. Grahovac, M. Soloski, G. Jung, and H.-G. Rammensee, submitted); thus, only a relatively small number of nonapeptides would fit to Qa–2. It appears, therefore, that at least some of the nonclassical class I molecules, like Hmt and Qa–2, are peptide receptors of narrower specificity than ordinary class I molecules.

HOW DO CELLS MAKE THE PEPTIDES?

This question can be answered by a plain "not known" (June 1992). However, there is a long list of observations that give hints towards the mechanisms involved. Consideration of some of these observations, and disregard of others, has led to a view now widely accepted although unproven.

The Common Dogma

A widely publicized view of the mechanism for peptide loading of class I molecules is the following (see 84–101). Proteins are cut into the correct

8-mers or 9-mers by proteasomes in the cytosol. The resulting peptides are then transported to the ER by ATP-dependent peptide transporters encoded by TAP 1 and TAP 2 genes located in the neighborhood of MHC class II genes. In the ER, class I molecules select the peptides fitting to the respective motifs and bring them to the cell surface. There is no doubt on the last step, i.e. that class I molecules transport peptides to the plasma membrane. There is also no doubt that TAP 1 and TAP 2 genes are essential for proper function of class I molecules (88, 94); indeed, the TAP gene products influence the peptide pattern that could be eluted from a rat class I molecule (100). The other parts of the dogma, including the compartmentalization, are merely speculative. There is no evidence so far that "peptide transporters" really do transport peptides, nor is there an indication on the identity of peptides potentially involved, although a good dozen of papers or editorials have been published in *Nature* or *Science* so far on those genes or molecules. Another explanation (although not very likely) is that the latter act as MHC-flippase, that is to toggle class I molecules from the ER lumen to the cytosol and back, as has been acknowledged in a recent review (99). The following paragraphs deal with observations that do not easily fit with the above model.

Protein But Not Final Size Peptide Is Found in the Cytosol

One of the first naturally processed T cell epitopes to be isolated (but not identified) was derived from β-galactosidase expressed from a transfected leaderless gene in P815 tumor cells (12). The transfectant, P13.1, contains high amounts of β-galactosidase in the cytosol, as detectable by β-galactosidase enzyme activity. However, the naturally processed peptide recognized by L^d-restricted T cells that can be found in whole cell extracts as well as in the membrane fractions, was not detected in the cytosol (12 and O. Rötzschke, K. Falk, H.-G. Rammensee, unpublished). Thus, if the final sized peptides are really produced in the cytosol, their duration or half-life in this compartment must be too short to allow detection.

Occurrence of Peptides in Whole Cell Extracts Is MHC Dependent

As mentioned above, whole cell extracts from β-galactosidase expressing P13.1 cells ($H\text{–}2^d$) do express the L^d-restricted β-galactosidase T cell epitope. EL4 tumor cells ($H\text{–}2^b$) transfected with the same gene and expressing even more β-galactosidase activity in the cytosol do not contain the L^d-restricted peptide (12 and K. Falk, O. Röztschke, H.-G. Rammensee, unpublished). This result indicated that coexistence of the correct class I molecule is required for occurrence of a given T cell epitope, since EL4 cells do not express L^d. This notion was confirmed with several

other antigens, including minor H and viral antigens (15, 16, 38, 55). MHC dependency of the cellular peptide content of cells was formally proven by comparing the peptides extracted from class I wild type and mutant mice (15) and was confirmed by analyzing cells transfected with class I genes (38, 102) as well as with tissue from transgenic mice (103). A semiquantitative study, using the K^b-restricted minor H peptide, H–4^b, indicated that a high copy number (5000-fold over detection limit) of K^b-restricted H–4^b peptide is present in an extract from 10^9 P815 cells transfected with K^b, but no detectable peptide is detected in untransfected P815 cells (102). These data suggest that, at a given point, not a single detectable copy of the final sized H–4^b peptide is present in cells not expressing K^b. An exception to the strict MHC dependency of peptide occurrence in whole cell extracts has been reported recently for one L^d-ligand recognized by alloreactive T cells (51).

Precursor/End Product Relationship?

The minor H antigen, H–4^b, mentioned above, shows a peculiarity informative for general considerations on antigen processing. In peptide mixtures eluted from purified K^b molecules of H–4^b expressing cells, T cells recognize but one H–4^b peptide (called H–4^b main peptide) (15). In contrast, however, H–4^b specific CTL recognize two peptides in whole cell extracts of K^b expressing H–4^b cells (15, 102). These two peptides can be distinguished by their behavior on HPLC; one of them is identical to the H–4^b main peptide, the other is called the "H–4^b prepeak." If whole cell extracts of cells lacking K^b are analyzed, only one H–4^b peptide is found, the H–4^b prepeak. Thus, the H–4^b main peptide is strictly MHC dependent, like most other class I restricted peptides (see previous paragraph), whereas the H–4^b prepeak peptide (being not a natural K^b ligand) is MHC independent in its occurrence in whole cell extracts. We speculated that the two peptides may be linked by a precursor/end product relationship (15). The MHC independent one could be a precursor peptide cut out from the H–4^b protein by enzymes that are independent of MHC molecules. This larger precursor—which happens to be able not only to bind to K^b but also to be recognized by K^b-restricted CTL—is then transported to the compartment of class I loading, binds to K^b, and then is trimmed to give the final sized ligand, according to our hypothesis (7, 15, 19). Alternatively, the MHC independent peptide might be derived from another proteolytic pathway unlinked to class I-restricted processing.

A similar situation—two peptides found in whole cell extracts, only one of which is MHC dependent and a natural class I ligand—was found for the peptide-specific, alloreactive, K^b-specific CTL clone 27.5 (16). In addition to an MHC-dependent peptide, this clone recognizes a peptide

present in spleen cells of all mouse strains, in human cells (Jurkat), and even in yeast or earthworm cells, indicating that this peptide—obviously derived from a conserved protein—is really MHC-independent.

Considering these data, one could speculate that the final size peptides—the class I ligands—are always derived from MHC-independent larger precursor peptides, and that detection of the latter depends on the chance that a given T cell happens to recognize not only the natural class I ligand, but also its larger precursor.

An alternative explanation for the observed MHC-dependency of peptide occurrence in cells, however, would still be "determinant protection" of entirely preprocessed peptides by binding to MHC molecules (15, 104, 105). Such a mechanism, however, would pose a principal problem that is discussed in one of the following paragraphs.

The Location of a Peptide Stretch Within a Protein Does Not Influence the Identity of Processed Peptides

The sequence coding for a natural L^d-ligand, YPHFMPTNL from murine cytomegalovirus immediate early protein, was inserted into several sites of an unrelated gene, with or without linker sequences, and the construct was expressed in cells (55). In all cases, the natural L^d-ligand was the same nonapeptide, although considerable quantitative differences were observed. In another study, when viral epitopes were inserted into various protein sites, the natural ligands were not identified, but recognition of all the different constructs by the same T cells suggested that the same epitope was processed in all cases (106). Thus, although the data base for these kind of experiments is still small, it appears that the identity of a processed class I ligand is generally independent of the flanking amino acid residues in the protein of origin, and that all the informations for cleavage sites must be within the ligand sequence. In still another study, certain flanking sequences of epitope-containing minigenes transfected into cells did not allow T cell recognition (107); because the identity of the corresponding ligands had not been identified, the data are still consistent with influence of flanking sequences on quantity but not identity of peptides.

Human Cells Know How to Make the Peptides for Mouse Class I Molecules

The alloreactive, K^b-specific CTL line 26T0–3 not only recognizes H–2^b mouse cells, but also human Jurkat tumor cells transfected with K^b (16). A peptide recognized by this CTL line can be isolated from both mouse and human cells expressing K^b. The peptide is probably derived from a protein conserved between mouse and human. Biochemical comparison of the peptide from mouse cells with that from human cells indicates that

both are identical (108). Another ligand, recognized by CTL clone 9.6, yielded similar data, thus confirming the notion.

Ovalbumin-specific, K^b-restricted CTL recognize the ovalbumine peptide SIINFEKL on K^b molecules of mouse cells (26), a point mentioned previously. Human HeLa cells transfected with both the ovalbumin gene and the K^b gene are also recognized by above CTL, and SIINFEKL is a natural ligand of K^b molecules of those HeLa transfectants (O. Rötzschke, K. Falk, N. Shastri, H.-G. Rammensee, submitted).

Thus, at least in those three examples, human cells process the same peptides from a given protein, if the relevant class I molecule, K^b in the examples given, is expressed in the cells. This indicates that the mechanism cutting proteins into peptides for class I loading—in its specificity—is conserved between different species. Moreover, this specificity is also the same between different tissues, and also between different mouse strains, since minor H peptides like H–4^b or H-Y appear to be the same no matter which tissue or mouse strain with correct MHC expression is analyzed (15, 103). Even tissue like brain tissue, normally almost class I negative (and thus, minor H peptide negative), can be induced to express the same H–4^b peptide as other tissue upon expression of transgenic K^b (103).

Extrapolating these data suggests that a single mouse brain cell, for example, is instantly able to provide any peptide ligand required not only by any of the hundreds of mouse class I molecules but also by the thousands of different class I molecules in humans and all other mammals. The commonly accepted model for antigen processing—postulating complete peptide processing before contact with class I molecules—would require a constant pool of ten of thousands of processed ligands fitting to all the class I molecules of all mammals, and this even in cells like brain cells that don't even express their own class I genes.

In addition to this conserved, nonpolymorphic general mechanism, however, polymorphism in peptide loading that has been shown for the cim system in rats (100) and suggested for B27 molecules in humans (109), might exist as an epiphenomenon. One possibility is also that the conserved mechanism works for all class I molecules requiring a hydrophobic C-terminus of peptide ligands, whereas the polymorphic mechanism works for class I ligands with a charged C-terminus, as for B27 ligands. (The B27 molecule appears to have a flexible pocket for the C-terminal peptide side chain able to accept either hydrophobic or charged residues; 110). Thus, the observed functional polymorphism might be due to the presence or absence of transacting factors (TAPs or proteasomes?) that are able to generate, transfer, or treat otherwise peptides terminated by charged residues.

A Model for Peptide Processing

Some of the above observations do not easily fit into the commonly accepted model on peptide processing. We have, therefore, proposed an alternative model that would explain those observations (7, 15, 19). The main point is that it assumes an instructive role for class I molecules in processing. We hypothesize that a protein is first degraded into (precursor-) peptides larger than the final class I ligand (Figure 1). This can be, for example, in the cytosol, but is also conceivable in other compartments. The endopeptidase(s) doing the cutting could have a specificity such that cleavage occurs C-terminal of hydrophobic (Met, Ile, Leu, Val, Phe) or charged residues (a specificity covered by proteasomes); this way the precursor peptides would already have the correct C-terminus fitting to most class I motifs known so far. The resulting peptide precursors—if not produced in the ER itself, like leader peptides would be, as we had suggested (16)—would then be translocated to the site of class I loading, that is, the ER or the early Golgi. This might be done by the putative ATP-dependent peptide transporters which might be specific for the respective C-terminus or size, although there is no evidence so far that these molecules

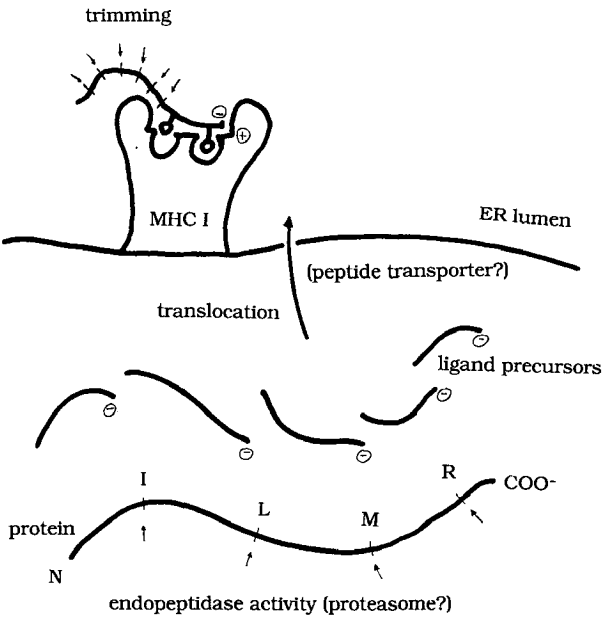

Figure 1 Model for antigen processing in the class I pathway. For explanation, see text.

transport peptides and, at least in in vitro systems, it appears that peptides can cross the membrane of microsomes without requiring ATP (111–113). The precursor peptide would then bind with low affinity to fitting class I molecules, for example, first by contacting a conserved positive charge (Lys 146) of the groove with the peptide's C-terminus, and then by insertion of anchor residue side chains into the allele specific MHC pockets. The N-terminus, still too long, would then be trimmed to the allele-specific length by an unknown peptidase activity that could be an exopeptidase. As soon as the final size of the peptide is reached, it would snap into the groove and be bound with high affinity.

Proof for natural binding of larger peptides to A2 molecules as well as their trimming down to nonamers (from both ends, however) has been convincingly demonstrated by the work of Henderson et al, and Wei & Cresswell, who looked at natural peptides derived from a leader sequence (70, 98; Table 1). This work, in addition, confirmed our earlier hypothesis that leader peptides generated in the ER can serve as class I ligands (16).

It is clear that most elements of the alternative model described in this paragraph are highly speculative; since the model, however, can explain some of the intriguing facets of class I–restricted antigen processing, it may be useful as a working hypothesis.

HOW MANY PEPTIDES ARE PRESENTED?

Copy Number of a Given Peptide

Comparing the amount of natural VSV-derived peptide RYQVYQGL found on K^b molecules on infected cells with a defined amount of synthetic peptide, it was estimated that about 5% to 10% of K^b molecules of an infected cell are occupied with the VSV peptide (18). If we assume that 10^5 K^b molecules are expressed on a cell, this would amount to 10,000 RYQVYQGL peptide copies per cell. On the other hand, the K^d-restricted peptide TYQRTRALV of influenza is found only in 200 to 500 copies per infected cell (14). Similarly, the D^b-restricted influenza peptide ASNENMETM occurs in about 200 copies per cell (14), and the K^b-restricted ovalbumin derived peptide SIINFEKL as well as the tumor-associated K^d-ligand KYQAVTTTL is found in around 100 copies per respective cell (26, 62). The prominent self-peptide SYFPEITHI from K^d-molecules of P815 tumor cells, representing the most abundant peptide species on K^d in this cell, is found in about 10,000 copies per cell (19). Thus, the copy number of individual peptides can be rather variable; judging from chromatography profiles of peptide pools eluted from class I molecules, it appears that on normal cells a few peptides are in the copy number range of SYFPEITHI, whereas most other peptides are at a much

lower rate, that is in the range of a couple of hundred copies. This fits well with mass spectroscopic analysis of A2-eluted ligands (21), and also fits well with the minimal number of synthetic peptides required to detect class I–restricted T cell recognition, if the peptides are added to the cells from outside (114, 114a).

The Number of Different Peptides per Cell

If a cell expresses 1 or 2×10^5 class I molecules of one kind, and the average copy number of a given peptide is 200, one would expect about 500 to 1000 different peptides per cell (14). Mass spectrometric analysis has allowed the detection of 200 different peptides eluted from A2 molecules, representing 50% of the A2-ligand population (21). Due to limits of detection, many low-copy number peptides could have been missed, and it was estimated based on the data that the number of peptides could exceed 1000. Thus it appears reasonable to assume that a given class I species presents around 1000 different peptides, with a rather broad range of copy numbers, however.

IMPLICATIONS FOR SELF-TOLERANCE

Self-Tolerance of Self-Peptides Is MHC restricted

The phrase "MHC restriction of self-tolerance" describes the events leading to the silencing of self-reactive T cell clones during their differentiation (115); they are dependent on the same kind of T cell recognition as in mature T cells, that is, recognition of MHC and antigen. Several years ago we learned that in bone marrow chimeras self minor H antigens do not induce tolerance if they are not presented with self MHC molecules, and normal mice do contain T cells reactive to self minor H antigens if presented on foreign MHC molecules (116–118). However, the antigens involved were not characterized at the molecular level, and the notion has been challenged (119). Peptide-specific alloreactive T cells allow us to readdress this question. The K^b-restricted CTL clone 27.B2, derived from a bm1 mouse (expressing K^{bm1}) recognizes a natural ligand of K^b that happens to be also a natural ligand of K^{bm1}. 27.B2 CTL recognize this peptide only if bound to K^b molecules, and not if bound to self K^{bm1} molecules (16, 120). Similar settings have been found for other alloreactive CTL (50). It can be concluded that T cells can distinguish if the same peptide ligand is presented by different class I molecules, and also that T cells in the bm1 mouse have not been negatively selected against the complex of self-peptide and foreign MHC molecule. Thus, negative selection of T cells during differentiation must require recognition of peptide ligands bound to MHC molecules.

T Cells Are Only Tolerant to Self Peptides That Are Actually Presented

Negative selection only of T cells recognizing self-peptides presented by MHC molecules would implicate that T cells are not at all tolerant to self-peptide not naturally presented on self MHC molecules. This notion was confirmed experimentally. B6 T cells could readily be stimulated with synthetic peptides according to stretches of a self-protein, β-microglobulin, to result in peptide-specific, class I–restricted CTL (78). Those peptides did not contain K^b or D^b-restricted motifs, and the CTL did not recognize H–2^b cells expressing β_2-microglobulin. Thus, the respective peptides are not presented naturally by cells but can bind to H–2^b molecules and induce CTL responses. Similar responses were obtained by stimulating T cells with trypsin-digested self-proteins (78). Thus, "self" for class I–restricted T cells is a rather limited selection of peptides derived from self-proteins, excluding the majority of self-protein sequences (14, 121). Consequently, peptides derived from that majority are treated as "foreign" by T cells, although derived from self sequences.

A COMPARISON WITH CLASS II

The approach of isolating and sequencing natural ligands of MHC molecules has also been applied for class II (67–69a). Differences distinguishing characteristics of class II ligands from those of class I are: (i) Class II ligands are longer, ranging roughly from about 12 to 20 residues. (ii) A given class II species can present peptides of various lengths, that is, strict allele specific length requirements are not observed. The class II peptide groove, therefore, appears to be open at both ends. (iii) Class II molecules appear to have allele-specific peptide motifs as well, although in the first publication on identified ligands, obvious motifs were not observed (67). More recent evidence indicates motifs containing two or three anchor-like positions that are not as stringent in occupancy requirements as is the case with class I molecules. A simplistic cartoon visualizing the principal differences between class I and class II ligands is in Figure 2.

CONCLUDING REMARKS

The function of MHC class I molecules is to display at the cell surface a selection of small peptides derived from cellular proteins. The selection is such that, on average, every cellular protein has a chance to participate with one or two peptides in this sampling (14, 121). Each member of a species displays her or his individual peptide selection, since the rules for

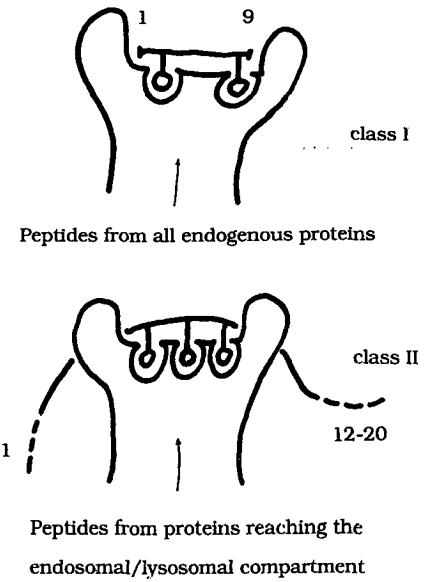

Figure 2 The principle differences between class I and class II ligands in a simplistic view.

peptide presentation are determined by the polymorphic MHC alleles (and in addition by the polymorphic *trans*-acting factors). An individual's T cell population is tolerant against his or her self-peptide collection, since all T cells recognizing one of these self-peptides are silenced during differentiation. If a new peptide appears, T cells can recognize it and attack the cell expressing it, eventually the death of the cell or necrosis of the tissue results. New peptides are likely to occur after a cell has been invaded by new genes, such as genes from viruses or other pathogens. Mutations in self-proteins could also lead to new peptides (122), although the chance that a point mutation hits a stretch carrying the motifs relevant for a cell is small. A third setting that may lead to occurrence of new class I ligands is the aberrant expression of proteins (60). In addition, the aberrant up-regulation of self-proteins could also lead to T cell recognition, since T cells can distinguish not only between different peptides but also between different quantities of the same peptide (55). Thus, peptides presented by class I molecules allow for control of the cell's interior through the immune system.

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THE IL–2 RECEPTOR COMPLEX: Its Structure, Function, and Target Genes

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KEY WORDS: IL–2 receptor β-chain (IL–2Rβ), signal transduction, cellular proliferation, tyrosine kinase activation, nuclear proto-oncogene induction

Abstract

Proliferation of T lymphocytes is triggered by the interaction of IL–2 with its specific receptor following T lymphocyte activation. The receptor for IL–2 consists of at least three distinct subunits, the α chain (IL–2Rα), the β chain (IL–2Rβ), and the γ chain (IL–2Rγ). Although the role of IL–2Rγ in IL–2 signalling remains unclear, IL–2Rβ is the subunit critical for receptor-mediated signalling. Because IL–2Rβ lacks any apparent catalytic motifs, IL–2Rβ may be physically or functionally coupled to other signalling molecules.

Structure-function studies of IL–2Rβ have revealed that at least two distinct cytoplasmic regions of IL–2Rβ are involved in IL–2–induced cellular signalling. The "serine-rich" region of IL–2Rβ was identified as a region critical for IL–2–induced mitotic signalling from experiments in which IL–2Rβ mutant cDNAs lacking a particular cytoplasmic region or regions were expressed in an IL–3–dependent mouse pro-B cell line (BAF-B03). Meanwhile, another cytoplasmic region of IL–2Rβ, the "acidic" region, is responsible for its physical association with an *src*-family protein tyrosine kinase (PTK), $p56^{lck}$ and is critical for activating the $p56^{lck}$ PTK following IL–2 stimulation.

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It is now evident that IL–2Rβ is linked to at least two intracellular signalling pathways that mediate nuclear proto-oncogene induction. One pathway is linked to tyrosine phosphorylation events, mediated by a *src*-family protein tyrosine kinase (PTK), and that pathway leads to the induction of the c-*fos*, c-*jun*, and other genes of this family. Another pathway leads to c-*myc* gene induction by an as yet unknown mechanism. We discuss the complex signalling machinery that links the cell surface receptor to the nuclear events.

INTRODUCTION

Interleukin–2 (IL–2), one of the first lymphokines to be identified, plays a central role in the clonal expansion of activated T lymphocytes (T cells) by interacting with its specific cell surface receptor (IL–2 receptor) (1–4). Antigen-specific, clonal proliferation of T cells is initiated via a process of signal transduction, wherein the specific interaction of the antigen/MHC molecule and T cell antigen receptor complex (TCR) triggers the expression of IL–2 and its homologous receptor (IL–2R). The interaction of IL–2 with IL–2R leads to the stimulation of a set of complex, still mostly unknown, signal transduction pathways resulting in cell proliferation (5–8).

In addition to its potent T cell growth-stimulatory activity, IL–2 mediates multiple biological processes, including growth and differentiation of B cells (9, 10), generation of lymphokine-activated killer cells (11, 12), augmentation of natural killer cells (13), and proliferation and maturation of oligodendroglial cells (14). Furthermore, recent studies have shown that IL–2 can act negatively in regulating cell growth, programming mature T cells for apoptosis (15), and reversing in vitro T cell clonal anergy (16). These observations suggest that IL–2 delivers various signals to a wide range of cell types via interaction with its receptor.

The specific cell surface receptor (IL–2R) that binds IL–2 is composed of at least three distinct polypeptides, the IL–2Rα, IL–2Rβ, and IL–2Rγ chains. The genes encoding IL–2 and these three receptor subunits have been cloned, and their complete primary structures have been deduced (17–22). In addition, evidence has accumulated that suggests the critical role of IL–2Rβ in IL–2 signal transduction (23–26), although the possible role of IL–2Rγ in IL–2 signalling remains unclear. In this article we first describe the molecular nature of the IL–2 receptor (IL–2R) and then provide an overview of our knowledge of IL–2 receptor–mediated signal transduction. Finally, we discuss the complex signal transduction machinery that connects stimulation of the cell surface receptor and nuclear events.

MOLECULAR NATURE OF THE IL–2 RECEPTOR (IL–2R)

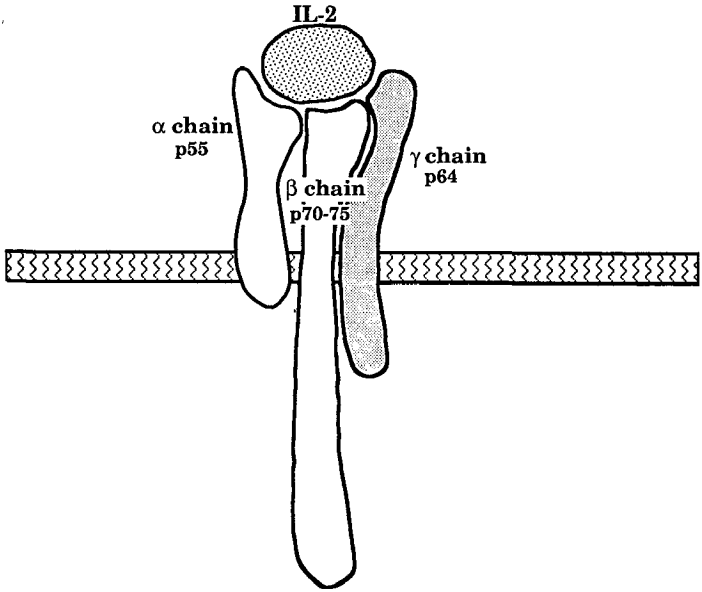
Three classes of IL–2Rs (high, intermediate, and low affinity) have been identified. The high-affinity (Kd = 10^{-11}M) IL–2R contains three distinct subunits: IL–2Rα, IL–2Rβ, and IL–2Rγ. The intermediate-affinity (Kd = 10^{-9}M) IL–2R contains two distinct subunits, IL–2Rβ and IL–2Rγ. In contrast, IL–2Rα alone binds IL–2 with low affinity (Kd = 10^{-8}). Figure 1 shows a schematic representation of the high-affinity IL–2R. Here we describe the molecular natures of the individual IL–2R subunits.

IL–2 Receptor α-Chain (IL–2Rα)

The biochemical characterization of the IL–2 binding component on cell surfaces was greatly facilitated by the development of a monoclonal antibody (designated as the anti-Tac antibody) which reacts with activated human T cells (27). The human IL–2Rα, originally described as the Tac antigen, was identified as a 55-kDa membrane glycoprotein (p55) capable of binding IL–2 (28, 29). Three groups employed similar strategies for protein purification and subsequent cDNA cloning of the human IL–2Rα (18–20). The deduced amino acid sequence of the human IL–2Rα predicts a mature protein of 251 amino acids (aa) with a signal peptide of 21 aa in length. The primary structure of the IL–2Rα shows no significant sequence homology with other known receptor molecules. IL–2Rα lacks structural features characteristic of members of the immunoglobulin superfamily and does not belong to the cytokine receptor superfamily. Within this chain, the amino-terminal 219 aa residues constitute the extracellular region, the next 19 aa residues, the membrane spanning region, and the carboxy-terminal 13 aa residues, the cytoplasmic region. When the cloned human IL–2Rα cDNA was transfected into nonlymphoid cells and lymphoid cells lacking IL–2–binding ability, it became evident that IL–2Rα constitutes only the low-affinity IL–2 binding form, a form that is nonfunctional with respect to IL–2 internalization and IL–2 signalling (30, 31). However, when the human IL–2Rα cDNA was introduced and expressed in several murine T cell lines (EL–4 and CTLL–2), the high-affinity as well as the low-affinity IL–2R were reconstituted on the cell surface, suggesting a contribution of the α-chain to the formation of the high-affinity receptor (30, 32, 33). Mutational analysis showed that the N-terminal 83 amino acid residues of the IL–2Rα chain, especially residues 1–6 and 35–43, were important for IL–2–binding function (34).

IL–2 Receptor β-Chain (IL–2Rβ)

Subsequently another component of IL–2R was biochemically identified by affinity cross-linking experiments (35–38). The structure of this com-



	α	αβ	αβγ	βγ	β	γ
Kd values [M]	*10^{-8}	*10^{-10}	10^{-11}	10^{-9}	*10^{-7}	no binding
Mitotic signaling	no	?	yes	yes	?	no

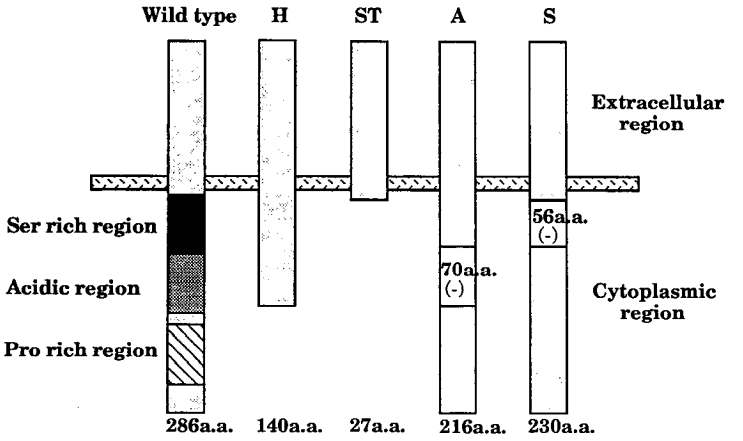
Figure 1 A schematic representation of the high-affinity receptor for IL–2. Noncovalently associated IL–2Rα/β/γ heterotrimer forms a high-affinity receptor for IL–2. The Kd values are indicated for the different combinations of subunits. The asterisks represent the Kd values measured on fibroblasts expressing each subunit or subunits.

ponent, IL–2Rβ (p75), was elucidated through the expression cloning of the cDNA for the human IL–2Rβ, using monoclonal antibodies against IL–2Rβ (21, 39). The full-length IL–2Rβ cDNA contains a large open reading frame that encodes a protein consisting of 551 amino acids (21). From the deduced structure of the protein, the NH2-terminal 26 amino acids apparently comprise the signal sequence, leaving 525 aa to make up the mature form of IL–2Rβ. Within this chain, regions of 214 aa, 25 aa,

and 286 aa in length constitute the extracellular, membrane-spanning, and cytoplasmic regions, respectively. Several unique amino acid motifs were found within the extracellular region of IL–2Rβ, and their resemblance to sequences found in other cloned cytokine receptors is discussed later. The cytoplasmic region of IL–2Rβ is far larger than that of IL–2Rα, but it does not contain any apparent catalytic motifs such as a kinase consensus sequence (40). It is rich in proline (24/286) and serine (30/286) residues and is notably biased for negatively charged amino acids. Thus, this region contains 40 negatively charged amino acids (glutamic and aspartic acids), but only 18 positively charged amino acids (lysine and arginine). This bias is particularly evident in the middle portion of this region (residues 345–390 aa). The cytoplasmic region of IL–2Rβ can be divided into three subregions based upon their amino acid compositions. These subregions have been designated as the "serine-rich" region, the "acidic" region, and the "proline-rich" region, as shown in Figure 2.

IL–2 Receptor γ-Chain (IL–2Rγ)

IL–2Rβ binds IL–2 with intermediate affinity (Kd = 10^{-9}M) when expressed in several types of cells such as large granular lymphocytes (LGL), oligodendrocytes, and T cells lacking IL–2–binding ability (21, 24, 26). However, IL–2Rβ binds IL–2 with extremely low affinity (Kd = 10^{-7}M) when expressed in fibroblasts such as NIH–3T3, L929, and COS–7 cells (21, 41, 42). In addition, IL–2 mutated at Glu–141 binds IL–2Rα and IL–2Rα/β heterodimers expressed on fibroblasts with normal affinity but is defective in binding to the same receptors on T cells. This mutant IL–2 also does not trigger the mitotic signal, suggesting the interaction of Glu–141 of IL–2 with an unidentified receptor component(s) (43). These results suggested that a cell-type specific component, now referred to as IL–2Rγ (p64), might be involved in the formation of the functional intermediate- and high-affinity IL–2Rs. IL–2Rγ (p64) can be co-precipitated with IL–2Rβ in the presence of IL–2 in lymphoid cells bearing the high-affinity IL–2R (44). Furthermore, the amount of IL–2Rγ coprecipitated with IL–2Rβ was proportional to the number of IL–2–binding sites in two T cell subclones, whereas the amount of IL–2Rβ was not correlated with the number of IL–2–binding sites in these subclones (45). Recently, the cDNA for the human IL–2Rγ chain has been cloned, and the complete nucleotide sequences of the cloned cDNAs have been determined (22). From the deduced structure of the protein, the NH2-terminal 22 aa apparently comprise the signal sequence, leaving 347 aa to make up the mature form of IL–2Rγ (22). Within this chain, regions of 232 aa, 29 aa, and 86 aa in length constitute the extracellular, membrane-spanning, and cytoplasmic



	cell growth	association with p56^{lck}	activation of p56^{lck} PTK by IL-2
Wild Type	+	+	+
H mutant	+	+	N.D.
ST mutant	−	−	N.D.
A mutant	+	−	−
S mutant	−	+	−

N.D. : not determined

Figure 2 Structure and biological properties of the wild-type and mutant IL–2Rβs. The cytoplasmic region of IL–2Rβ can be tentatively divided into three subregions as shown in this figure.

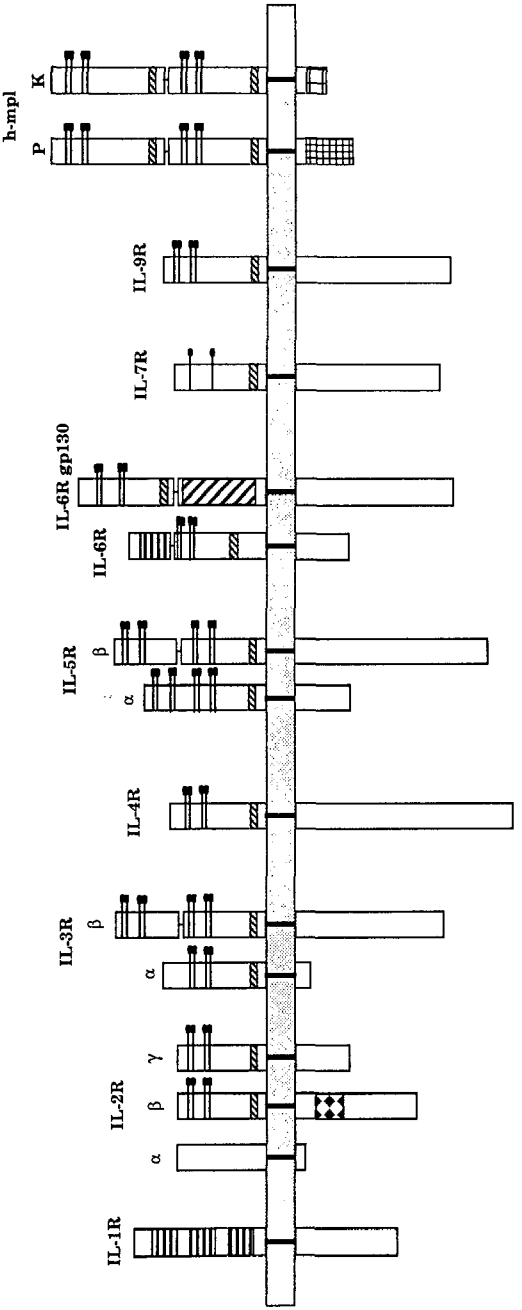
regions, respectively. The cytoplasmic region of IL–2Rγ is considerably shorter than that of IL–2Rβ. Interestingly, sequence from positions 288 to 321 appears to be homologous to the *Src* homology region 2 (SH2), but it does not contain any apparent catalytic motifs (22). The predicted

extracellular domain of IL–2Rγ contains several unique motifs that are found in other cytokine receptors, indicating that IL–2Rγ belongs to the cytokine receptor superfamily. When the cDNAs for IL–2Rα, IL–2Rβ and IL–2Rγ were introduced and expressed in fibroblasts, the high-affinity receptor for IL–2 was indeed reconstituted (22). In addition, it appears that IL–2Rγ is required for the receptor-mediated internalization of IL–2 (22). Other components associated with IL–2R have also been proposed based on co-immunoprecipitation of molecules that can be chemically cross-linked with IL–2R; their biochemical structure and function remain undefined (46–51).

CYTOKINE RECEPTOR SUPERFAMILY

Recently, a number of cytokine receptors have been cloned. They share significant homology and constitute a novel family of receptors (52–57). Their primary sequences distinguish them from the previously characterized growth factor receptors. Figure 3 shows a schematic representation of the members of this receptor superfamily. The family includes IL–2Rβ, IL–2Rγ, IL–3R (α and β-chain), IL–4R, IL–5R (α and β-chain), IL–6R and IL–6R gp130, IL–7R, IL–9R, ciliary neurotrophic factor R (CNTFR), prolactin R (PLR), growth hormone R (GHR), erythropoietin R (EPOR), GM-CSFR (α and β-chain), G-CSFR and leukemia inhibitory factor receptor α-chain (LIFRα) (58, 59). Interestingly, the human high-affinity receptors for GM-CSF, IL–3, and IL–5 share a common β subunit. The mouse IL–3 receptor includes either AIC2A or AIC2B as a β subunit, while the mouse GM-CSF and IL–5 receptors share the common β subunit (AIC2B) (60–64).

All the members of this family (except CNTFR) are type I membrane glycoproteins with a single hydrophobic transmembrane domain, oriented with their N-termini exterior to the plasma membrane. This extracellular domain contains two major regions of homology: One is a region containing four Cys residues located in the N-terminal half of the extracellular domain (Cys-X9–10-Cys-X-Trp-X26–32-Cys-X10–15-Cys). Disulfide bonds reportedly could be formed between the first and the second Cys residues or between the third and the fourth Cys residues located in the N-terminus of the GHR (55). Another sequence homology is Trp-Ser-X-Trp-Ser, which we refer to as the "WS motif" located proximal to the membrane-spanning domain. Based on secondary-structure predictions, this "WS motif" may reside in the floor of a crevice lying between two β strands common to these cytokine receptors. The "WS motif" may function as the main ligand binding site in these cytokine receptors (53). In the case of IL–2Rβ, the two Trp residues in the "WS motif" play a crucial



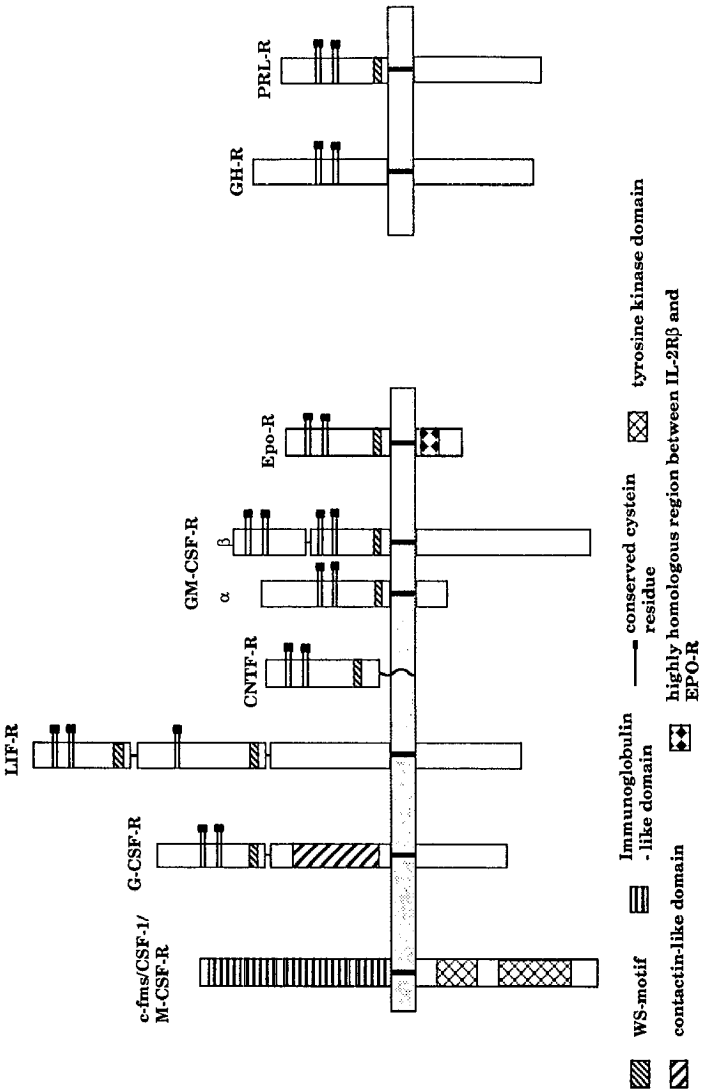


Figure 3 A schematic representation of the cytokine receptor superfamily.

role in the proper folding of the extracellular domain and in growth signal transduction mediated by IL–2Rβ (65). The ligand binding segment (approximately 200 amino acid residues) of the cytokine receptors is composed of two discrete folding domains which share significant sequence and structural similarities (53, 54). The C-terminal half of the extracellular domain shows an evolutionary linkage to the fibronectin type III modules found in a series of cell surface molecules with adhesive properties. It remains to be clarified whether the domains of this receptor family will indeed be involved in ligand binding or association with other extracellular structural components. As noted one component of the IL–2 receptor complex, IL–2Rα, does not belong to the cytokine receptor superfamily.

In addition to the structural similarities among the extracellular domains of the cytokine receptors, several conserved motifs in the cytoplasmic domains of the receptors have been reported. Three amino acid stretches are highly conserved between the G-CSFR and the IL–6R gp130, and one or two of them are localized within the cytoplasmic region of these receptors critical for growth signal transduction (66, 67). Furthermore, one of these amino acid stretches (box I) can be found in the cytoplasmic region of the human IL–2Rβ, mouse IL–3Rβ, mouse IL–4R, human IL–7R, human GM-CSFRβ, mouse EPOR, the recently identified oncogene v-*mpl* and its human counterpart, h-*mpl* (68, 69). These similarities in the structures of the cytokine receptors imply that some, if not all, members of this superfamily utilize common or closely related mechanisms to mediate signal transduction.

IL–2R–MEDIATED SIGNAL TRANSDUCTION

Structure-Function Relationships of IL–2Rβ

To elucidate further the role(s) of IL–2Rβ in IL–2–mediated signal transduction, it was necessary to develop a cDNA expression system that would allow us to study the function of the human IL–2Rβ upon IL–2 stimulation. The human IL–2Rβ cDNA was inserted into an expression vector and subsequently introduced and expressed in a mouse IL–3–dependent pro-B cell line, BAF-B03, a subclone of BA/F3 (70–72). BAF-B03 cells express endogenous mouse IL–2Rα at a relatively high level and exhibit low affinity binding for IL–2, but they fail to proliferate in response to IL–2. Clones expressing the human IL–2Rβ displayed both high and low affinities for IL–2 and became responsive to IL–2 (25). Similar results were also obtained when the human IL–2Rβ cDNA was introduced and expressed in a mouse IL–3–dependent mast cell progenitor cell line, IC–2 (73). These observations demonstrate that IL–2Rβ plays a critical role in transducing the IL–2–induced mitotic signal(s) in hematopoietic cell lines.

Furthermore, they suggest that the intracellular growth signal transduction induced by cytokines such as IL–2 and IL–3 may be mediated by common or closely related mechanisms in a wide range of hematopoietic cells.

In order to delineate and identify the region(s) of IL–2Rβ critical for growth signal transduction, a series of mutant IL–2Rβs with deletions in their cytoplasmic domains were generated and introduced into BAF-B03 cells (25). The structures and functional properties of the wild-type and a series of mutant IL–2Rβ chains are depicted schematically in Figure 2. The cDNA of pLCKRβ-H encodes the human IL–2Rβ with a deletion of 147 aa from the carboxyl terminus ("H-mutant"). The cDNA of pLCKRβ-ST codes for mutant IL–2Rβ, that retains only 27 aa of the cytoplasmic domain ("ST-mutant"). The cDNAs of pLCKRβ-A and -S encode IL–2Rβ molecules lacking the internal "acidic" region (313–382 aa) and the "serine-rich" region (267–322 aa) ("A-mutant" and "S-mutant"), respectively. The resultant transformant clones express epitopes on their cell surfaces recognized by an anti-IL–2Rβ antibody (Mikβ–1). Affinity cross-linking experiments using radioiodinated IL–2 (^{125}I-IL–2) confirmed that each of these transformants expressed mutant IL–2Rβ molecules of the expected sizes. Binding studies revealed that all the transformants expressed high affinity IL–2R, demonstrating that the truncations and internal deletions of the cytoplasmic domain of IL–2Rβ did not affect IL–2 binding. The transformants were further examined for their IL–2–dependent growth properties. Clones expressing the H-mutant responded to IL–2, but the clones expressing the ST-mutant failed to respond. Clones expressing the A-mutant also responded to IL–2, but less well than clones expressing the wild type IL–2Rβ. None of clones expressing the S-mutant were capable of responding to IL–2. It is noteworthy that the primary sequence of the "serine-rich" region of the human IL–2Rβ reveals about 80% homology with that of the murine IL–2Rβ (74), and that this region shows the high degree of homology with the corresponding region of the murine erythropoietin receptor (EPO-R) (75). Mutant EPO-R lacking this homologous region fails to respond to EPO (75).

These observations suggest that this "serine-rich" region of IL–2Rβ is important for mediating growth signal transduction. In the case of IL–2Rβ-mediated signal transduction, it is likely that other intracellular signal transducing molecules somehow couple to IL–2Rβ, since IL–2Rβ by itself does not have any apparent catalytic activity. It should be noted that the "serine-rich" region contains a conserved stretch of hydrophobic amino acids. Replacement of leucine 299 in this stretch with proline may induce a conformational change in IL–2Rβ, because the resultant receptor is no longer capable of transmitting the IL–2–induced mitotic signal (76). The amino acid residues surrounding this leucine residue are conserved among

the human and the murine IL–2Rβs and the murine EPO-R (21, 74, 77). Analysis by the Chou-Fasman program (78) predicts that the leucine299 residue would be localized within an α-helical structure within this region of IL–2Rβ. Assuming that this presumed helical structure within the "serine-rich" region of the IL–2Rβ chain has an important role in the molecular interaction(s) required for signal transduction, substitution of this leucine residue by proline might disrupt helix formation, resulting in the failure of certain signal transducing molecule(s) to associate with IL–2Rβ.

IL–2R–Coupled Tyrosine Kinases

Tyrosine phosphorylation of cellular substrates is believed to play a crucial role in the intracellular signal transduction pathways that regulate cellular activation and differentiation (79, 80). Accumulating evidence suggests that tyrosine kinase(s) are involved in signal transduction mediated by cytokine receptors (66, 75, 81–92). Engagement of these receptors results in the increased tyrosine phosphorylation of cellular substrates including, in many instances, the receptors themselves. Furthermore, a similar pattern of tyrosine phosphorylated cellular substrates has been observed following stimulation by several different cytokines, leading us to speculate that these signals are mediated by a common mechanism.

IL–2 stimulation results in the increased tyrosine phosphorylation of cellular substrates, including IL–2Rβ itself, presumably by activating a tyrosine kinase(s). An understanding of this activation requires the identification of the responsible kinase. In fact, it has been shown that a tyrosine kinase activity could be co-immunoprecipitated with IL–2Rβ (93, 94).

Currently, several members of the *src*-family protein tyrosine kinases (PTKs) have been shown to associate physically with several surface antigens (receptors). Documented examples include $p56^{lck}$ with the CD4/CD8, $p59^{fyn}$ with the T cell antigen receptor, $p56^{lyn}$ with the B cell antigen receptor and $p59^{fyn}$, $p56^{lyn}$ and $p62^{yes}$ with CD36 (95–99). These receptor-kinase molecular complexes may represent a novel functional unit for transmembrane signalling.

Among the *src*-family PTKs, $p56^{lck}$ was an attractive candidate as a mediator of IL–2Rβ-mediated signalling because of its abundance in T cells. Indeed, increased tryosine kinase activity and modification (serine/threonine phosphorylation) of $p56^{lck}$ following IL–2 stimulation has been reported (93, 100).

Direct evidence for a physical interaction between IL–2Rβ and $p56^{lck}$ has been reported recently (93). It was first demonstrated in the NK-like cell line, YT. Anti-IL–2Rβ antibody (Mik-β1) immunoprecipitates of YT cell lysates were shown to contain $p56^{lck}$ when examined by an immunoblotting analysis using anti-*lck* antiserum. In contrast control antibodies,

anti-Tac antibody and anti-CD7 antibody, failed to co-precipitate $p56^{lck}$. This association was further assessed in COS cells in which wild-type or the previously described mutant IL-2Rβ cDNAs were co-transfected along with *lck* cDNA and their molecular interactions were subsequently evaluated. The results, summarized in Figure 2, showed that the cytoplasmic region of IL-2Rβ containing the "acidic" region is primarily responsible for the molecular interaction with $p56^{lck}$. About 10 to 20% of the total IL-2Rβ molecules are estimated to be associated with $p56^{lck}$ in cells containing abundant amounts of the latter molecule. Conversely, only a small fraction (about 0.5–1%) of total cellular $p56^{lck}$ is physically associated with IL-2Rβ. The region responsible for the association with $p56^{lck}$ ("acidic" region) can be separated from an additional region responsible for mitotic signalling ("serine-rich" region); likewise, a domain of $p56^{lck}$ responsible for the association with IL-2Rβ has been identified. The *lck* tyrosine kinase can be divided into three domains: the N-terminal CD4 or CD8 binding domain, the modulatory domain containing the *src* homology sequences SH2 and SH3, and the kinase domain. The N-terminal half of the kinase domain is responsible for its association with IL-2Rβ.

In view of the structural similarity between the catalytic domains of *lck* and other *src*-family PTKs (40, 101), it was thought that IL-2Rβ might also be able to interact with other *src*-family PTKs. IL-2 is known to function in B cells (9, 102, 103) which express $p56^{lyn}$ and $p55^{blk}$ as well as small amounts of $p56^{lck}$. In fact, $p59^{fyn}$ physically associates with IL-2Rβ in BAF-B03 cells expressing IL-2Rβ at high levels, and in which $p56^{lck}$ is not detectable (N. Kobayashi, T. Kono, M.Hatakeyama, Y. Minami, T. Miyazaki, R. M. Perlmutter, T. Taniguchi, manuscript now in press). Furthermore, two *src*-family PTKs, $p59^{lyn}$ and $p56^{lyn}$, are activated following IL-2 stimulation of the above cells (N. Kobayashi, T. Kono, M. Hatakeyama, Y. Minami, T. Miyazaki, R. M. Perlmutter, T. Taniguchi, manuscript now in press). In agreement with the above results, activation of *lyn* reportedly is induced by IL-2 in F7 cells (104), a BAF-B03–derived transfectant cell line expressing the human IL-2Rβ (25). Therefore, there may be redundant and hierarchical associations with or activation of the *src*-family PTKs in a cell lineage–specific manner. It is also possible that other members of the cytokine receptor superfamily may interact directly with members of the *src*-family PTKs to transmit their signals, since activation of both $p59^{fyn}$ and $p56^{lyn}$ PTKs was equivalently induced by IL-2 or by IL-3 in the above cells.

Following IL-2 or IL-3 stimulation of BAF-B03 cells expressing IL-2Rβ at high levels, an apparent increase in the tyrosine phosphorylation of an 85 kDa substrate (pp85) as well as a limited number of other substrates was observed (Y. Minami, T. Kono, K. Yamada, N. Kobayashi,

A. Kawahara, R. M. Perlmutter, T. Taniguchi, ms. in prep.). pp85 was found exclusively in the cytosolic fraction and seems to be different from the modulatory subunit (p85) of the phosphatidyl inositol 3-kinase (PI3-kinase) which is a potential signal modulator in the IL–2 system (105–107). Because pp85 appears to be a major target of $p56^{lck}$ PTK following both IL–2 and IL–3 stimulation, understanding a role for pp85 in regulating cellular function will be crucial to unravelling this complex tyrosine kinase pathway. Cytokine-mediated signalling through IL–2 and IL–3 receptors may be similar or identical in respect to the induction of pp85 tyrosine phosphorylation. However, the IL–2 and IL–3 signal transduction pathways reportedly must be at least partially distinct in a mouse IL–3–dependent myeloid progenitor cell line, 32D, expressing the human IL–2Rβ (108). IL–2 induces phenotypic changes in these cells not induced by IL–3, including the up-regulated expression of IL–2Rα and IL–2Rβ and an increase in cell size.

Functional Domains of IL–2Rβ Required for IL–2–Induced Cellular Tyrosine Phosphorylation

As already mentioned, stimulation of BAF-B03 cells expressing IL–2Rβ by IL–2 increases tyrosine phosphorylation of cellular substrates, including pp85. To identify the functional domain(s) of IL–2Rβ responsible for this event, BAF-B03 cells expressing the wild-type or mutant IL–2Rβs (A- and S-mutants) at high levels were established. Interestingly, both the "acidic" and the "serine-rich" regions of IL–2Rβ are also required (Y. Minami, T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Perlmutter, T. Taniguchi, manuscript in preparation).

Stimulation of T cells with IL–2 rapidly activates $p21^{ras}$, a member of the signal transducing GTP-binding proteins (109). Both the "acidic" and the "serine-rich" regions of IL–2Rβ are required for this activation (T. Satoh, Y. Minami, T. Kono, T. Taniguchi, and Y. Kaziro, manuscript in press). Because herbimycin A, a specific inhibitor of tyrosine kinases, can block the accumulation of the active $p21^{ras}$/GTP complex induced by IL–3 or by GM-CSF stimulation of PT18 mast cells (110), $p21^{ras}$ activity appears to be regulated by tyrosine kinase(s).

Possible Mechanism for $p56^{lck}$ PTK Activation

As noted approximately 0.5–1% of cellular $p56^{lck}$ physically associates with IL–2Rβ in T cells, and IL–2 increases the tyrosine kinase activity of $p56^{lck}$ when measured in vitro using enolase as an exogenous substrate (93). Because of its low stoichiometric association, it is important to determine whether association of $p56^{lck}$ with IL–2Rβ is required in the tyrosine kinase pathway or whether it is merely a "by-stander." For this

purpose, BAF-B03 cells that express both IL–2Rβ (either the wild-type or mutant forms—A- S-mutants) and p56^{lck} at high levels were established (Y. Minami, T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Perlmutter, T. Taniguchi, ms. in prep.). In BAF-B03 cells expressing the wild-type IL–2Rβ along with p56^{lck}, a physical association between IL–2Rβ and p56^{lck} was indeed observed. Quantitative analysis revealed that only 1% of the total cellular p56^{lck} associates with IL–2Rβ in the above cells but that the amount of p56^{lck} PTK activated upon IL–2 stimulation exceeds the estimated amount of p56^{lck} associated with IL–2Rβ. A-mutant BAF-B03 cells expressing an IL–2Rβ lacking the "acidic" region essential for the association with p56^{lck} fail to induce the p56^{lck} PTK activation upon IL–2 stimulation (see Figure 2). This suggests that the association of p56^{lck} with IL–2Rβ, although representing only a small fraction of total p56^{lck}, is critical for the IL–2–mediated activation of cellular p56^{lck} PTK. Another mutant IL–2Rβ (S-mutant) lacking the "serine-rich" region—a region critical for transducing the IL–2–induced proliferative signal—also fails to activate p56^{lck} PTK (see Figure 2). Therefore, the physical association of IL–2Rβ with p56^{lck} appears to be necessary, but presumably not sufficient for activation of p56^{lck} PTK.

OTHER BIOCHEMICAL EVENTS IN IL–2 SIGNALLING PROCESS

One early biochemical event that follows IL–2 stimulation is a rapid increase in intracellular pH as a result of the activation of a Na^+/H^+ antiport (111). The involvement of some other well-characterized biochemical pathways implicated in cell proliferation, such as activation of protein kinase A (PKA) or protein kinase C (PKC) or Ca^{++} mobilization, seems unlikely (112–115). In addition to the tyrosine phosphorylation of cellular substrates, phosphorylation of serine and threonine of various substrates, including IL–2Rβ itself, results from stimulation with IL–2 (116). One serine/threonine kinase that is an attractive candidate in mediating the IL–2 growth signal is the ubiquitous cytoplasmic kinase p70–75, *Raf*–1. Indeed, engagement of the IL–2, IL–3, or GM-CSF receptors by their respective ligands results in the activation of *Raf*–1 kinase, possibly as a result of phosphorylation on its serine, threonine, or tyrosine residues (117–119). In the case of the IL–2 system, *Raf*–1 is the earliest cellular substrate so far identified that is phosphorylated by a tyrosine kinase(s) upon IL–2 stimulation. Because *Raf*–1 rapidly undergoes tyrosine phosphorylation and p56^{lck} rapidly undergoes serine/threonine phosphorylation upon the engagement of the IL–2 receptor, the activity of these two kinases may be mutually regulated. The important question of

whether $p56^{lck}$ is a substrate for *Raf*–1 kinase following IL–2 stimulation has not yet been addressed.

Stimulation of T cells by IL–2 causes a rapid increase in PI3-kinase activity in both cellular extracts and antiphosphotyrosine immunoprecipitates (105–107). A monoclonal antibody (Mik-β3) that recognizes a non-IL–2 binding epitope on IL–2Rβ specifically coprecipitates the PI3-kinase activity following IL–2 stimulation (105). Thus, it may be possible to identify the functional domain(s) of IL–2Rβ responsible for its association with or activation of PI3-kinase.

Another possible signalling mechanism involves the hydrolysis of glycosyl-phosphatidyl inositol (GPI). IL–2 stimulation provokes the rapid hydrolysis of GPI, generating two potential signal mediators, a glycosylated diacylglycerol and an inositol phosphate glycan (103, 120). Interestingly, IL–4 stimulation fails to induce this GPI hydrolysis in the same cell. Although the functional roles of these mediators have not yet been characterized, it is possible that a GPI-specific phospholipase might be activated after IL–2 receptor engagement.

IL–2 INDUCTION OF NUCLEAR PROTO-ONCOGENES

Nuclear proto-oncogenes are potentially critical targets for proliferative signals mediated by growth factor receptors (121, 122). Most of the nuclear proto-oncogene products manifest DNA binding properties individually or in combination with others, thereby functioning as regulators of gene transcription (123). Cytokines induce the expression of several genes, including nuclear proto-oncogenes, in T lymphocytes (124–126), B lymphocytes (127), and myeloid cells (128). Expression of these genes is not restricted to immuno-hematopoietic cell lineages but rather seems to involve the proliferative processes in a wide range of cell types. Following IL–2 stimulation, there is a rapid and transient increase in the number of c-*fos* transcripts, as well as a more stable accumulation of c-*myc* and c-*myb* transcripts (124–126, 129–132).

Two Signal Transduction Pathways Mediate Nuclear Proto-Oncogene Induction

IL–2Rβ is linked to at least two intracellular signalling pathways mediating nuclear proto-oncogene induction (133). One pathway involves tyrosine phosphorylation events, mediated by some *src*-family PTK(s), and leads to the induction of the c-*fos*, c-*jun* and other genes of this family presumably through the activation of $p21^{ras}$ (Y. Minami, T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Perlmutter, T. Taniguchi, ms. in prep.;

T. Satoh, Y. Minami, T. Kono, T. Taniguchi, Y. Kaziro, manuscript now in press). Another pathway leads to c-*myc* gene induction by an as yet unknown mechanism (133). Our current model depicting these IL–2R–mediated signalling pathways is shown in Figure 4.

In the case of c-*fos* gene induction mediated by IL–2Rβ, the serum responsive element (SRE) in the c-*fos* promoter is the primary target for the signal (131, 132). Induction of the c-*fos*/c-*jun* genes seems to be mediated by a tyrosine kinase pathway via the activation of p21^{ras}, since these cellular events (tyrosine kinase (*src*-family PTK) activation, p21^{ras} activation and induction of the c-*fos*/c-*jun* genes) induced by IL–2 require the identical cytoplasmic regions (both the "acidic" and the "serine-rich" regions) of IL–2Rβ (Y. Minami, T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Perlmutter, T. Taniguchi, manuscript in preparation; T. Satoh, Y. Minami, T. Kono, T. Taniguchi, Y. Kaziro, manuscript in press).

On the other hand, the induction of the c-*myc* gene in BAF-B03 cells correlates with the ability of cells to undergo mitosis (133). Indeed, BAF-B03 cells expressing the mutant IL–2Rβ (S-mutant) lacking the "serine-rich" region, a region critical for transducing the mitotic signal(s), fail to induce the c-*myc* gene. In contrast, BAF-B03 cells expressing another mutant IL–2Rβ (A-mutant) which retains the "serine-rich" region are still capable of inducing both c-*myc* gene expression and cellular proliferation.

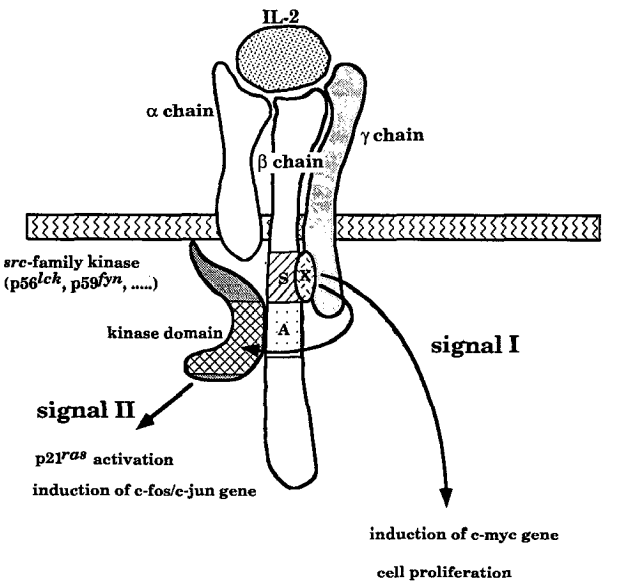


Figure 4 IL–2 receptor-mediated signalling involves at least two distinct pathways.

Hence, the "serine-rich" region of IL–2Rβ may elicit an as yet unidentified signal that leads to c-*myc* gene induction. Induction of the c-*myc* gene also correlates with the efficient induction of certain cell cycle genes, believed to be critical for cells to enter G2/M phase, including cyclins A and B, and cdc2 kinase. When BAF-B03 cells expressing the human epidermal growth factor (EGF) receptor are stimulated by EGF, they begin to enter S phase but fail to progress to G2/M (133). This deficiency in cell cycle progression can be rescued by ectopic expression of the c-*myc* gene, indicating a novel role for the c-*myc* gene in the S to G2/M transition of the cell cycle (133). These results suggest that c-*myc* functions upstream of certain cell cycle genes, regulating them either directly or indirectly.

CONCLUDING REMARKS

Recombinant DNA technology has made possible a rapid advance in our understanding of the structure of cytokines and cytokine receptors, including IL–2 and its receptor (IL–2R). In this review, we have discussed the salient features of IL–2R–mediated signalling. We have shown that IL–2Rβ is linked to at least two intracellular signal transduction pathways that mediate nuclear proto-oncogene induction. However, much remains to be elucidated before we will be able to fully understand the signal transduction pathways that activate target genes in the nucleus (c-*fos*, c-*jun* and c-*myc* genes etc) in response to stimulation of IL–2Rβ on the cell surface. For example, it is important to identify the molecule or molecules that couple IL–2Rβ activation to induction of c-*myc* genes. Apparently, IL–2 signalling is a complex process involving a large cast of molecules. We have discussed four molecules that are attractive candidates for transducing elements involved in this process ($p56^{lck}$, $p21^{ras}$, *Raf*–1 and PI3-kinase), but we do not know the exact roles of these molecules in the signal transduction pathway. For example: (i) What are the respective functions of these molecules? (ii) How are they activated? (iii) What are the identities and functions of their targets? We need to answer these questions in order to be able to describe a more complete scenario of IL–2 signal transduction.

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THE MOLECULAR DESCENT OF THE MAJOR HISTOCOMPATIBILITY COMPLEX

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Abstract

In the last few years, more than 500 primate major histocompatibility complex (*Mhc*) genes or parts thereof have been sequenced. The extraordinary sequence information is used here to draw conclusions about the manner of *Mhc* evolution. The *Mhc* genes are found to evolve at a relatively slow rate with the regularity of a clock. It takes from 1 to 6 million years for a new mutation to be incorporated into an *Mhc* allele, and the mutation rate is comparable to that of most other primate genes. The nonsynonymous sites coding for the peptide-binding region (PBR) are under relatively weak positive selection pressure (selection coefficient of a few percent only); the nonsynonymous non-PBR sites are under moderate negative selection pressure. The positive pressure is probably provided by parasites and is responsible for the trans-species persistence of allelic lineages at functional *Mhc* loci for more than 40 million years.

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THE BEL CANTO OF EVOLUTIONARY BIOLOGY

At the beginning of the nineteenth century, Italian opera composers rediscovered the charms of *bel canto*, a style introduced some 200 years earlier to accentuate the splendor of the human voice. In the *bel canto* operas of Bellini or Donizetti, the vocalist is often heard virtually alone, accompanied only by the simplest phrasing of a complex orchestra. Yet the effects are stupendous. Anybody who can hear Luciano Pavarotti singing "*Prendi: l'anel ti dono che undi che undi recavaal l'ara.*" in *La sonnambula* without his heart swelling with emotion must have lost his capacity for enchantment. In that graceful, lyrical melody is Beauty in her purest and simplest form.

Evolutionary biology has discovered its *bel canto* in molecular population genetics. Just as beautiful singing is the backbone of a Bellini opera, so do elegant mathematical concepts provide the sustenance of modern evolutionary theory. Like the melodies, the concepts are often cunning and deceptively simple. It is hard not to be impressed, for example, by the deduction that if in a population containing N breeding individuals a mutation occurs in a particular gene of a particular individual, then the probability that eventually all the individuals in the population will carry this mutation is $1/(2N)$, and the average time needed for this to happen is $4N$ generations. Grace, beauty, and simplicity are the hallmarks of molecular population genetics, simplicity in particular; it often hides the high mathematics that went into the derivation of a seemingly humble formula.

Since it is, not surprisingly, molecular population genetics that is unlocking the last doors to the last hidden secrets of the major histocompatibility complex, we believe it proper to devote this discourse to the description of some of its simple concepts and their application to the study of the *Mhc*.

PRIMER IN MOLECULAR EVOLUTION

Consider the nucleotide sequence of a gene at a particular locus from species A and another sequence of a gene at a homologous locus from species B. Align the two sequences so that identical nucleotides occupy corresponding *sites* and identify the differences (*nucleotide substitutions*). In the simplest situation, each substitution is the result of a mutation that occurred in one of the genes after the separation of the two species from a common ancestor. The number of substitutions divided by the total number of sites is the *genetic distance*, d, between the two genes, that is, the number of substitutions per site. For example, if each of the two

sequences is 300 nucleotides long and the two sequences differ by two substitutions, the distance between the two genes is $d = 2/300$ or 0.007. In other words, in each of the two genes there are 0.003 substitutions per site.

Nucleotide substitutions, however, come in two flavors: synonymous (those that do not change the amino acid sequence in the corresponding protein) and nonsynonymous (those that result in amino acid replacement). It is often prudent to treat synonymous and nonsynonymous substitutions separately so that we then have two kinds of distances, *synonymous* (d_s) and *nonsynonymous* (d_N). Assume that in our example one substitution is synonymous and the other nonsynonymous and that of the 300 sites, 70 are synonymous and 230 are nonsynonymous; for information on which site to consider synonymous and which nonsynonymous, see Li & Graur (1). The distances are then $d_s = 1/70$ or 0.014 and $d_N = 1/230$ or 0.004.

The calculation of distances is this simple only when the two species are closely related and the genes that are being compared diverged recently. When the separation times are long, one has to consider the possibility that two or more mutations occurred at the same site (*multiple hits*) and that a second mutation might have restored the original nucleotide at a given site (*backward* or *reverse mutations*). Several correction formulas are available that take these possibilities into account (2).

Suppose now that the same gene has been sequenced from several individuals representing different populations of species A, that the same is true for species B, and that always the same two substitutions have been found in the interspecies comparisons. We are then justified to extrapolate from the sample onto the entire species and to conclude that all individuals of one species differ from all individuals of the other species by these substitutions. How did this situation arise? Presumably, each substitution originally appeared as the result of a mutation in a single individual and then spread through the population until it ultimately replaced all the original forms of the gene—it became *fixed.* The spreading took place over many generations in the period since the separation of the two species. The two mutations could have occurred in one of the two species, or one mutation could have occurred in each species. To decide which of the two possibilities holds true, we would have to have the sequence of the gene from a species ancestral to both A and B, but for our present purpose, we do not need to know this. If we know the separation time of species A and B from the fossil record, we can calculate the rate at which substitutions accumulate at this particular locus. This *substitution rate*, K, is then the number of substitutions per site per unit of time. If, in our example, species A and B separated two million years (my) ago, the total substitution rate

is $K = 2/300/(4 \times 10^6) = 1.67 \times 10^{-9}$ per site per year, the synonymous substitution rate is $K_s = 1/70/(4 \times 10^6) = 3.6 \times 10^{-9}$ per synonymous site per year, and the nonsynonymous substitution rate is $K_N = 1/230/(4 \times 10^6) = 1.1 \times 10^{-9}$ per nonsynonymous site per year. (The reason for using the value of 4×10^6 is that substitutions had 2 million years (my) from the ancestor to species A and another 2 my from the ancestor to species B to accumulate.) Since accumulation of substitutions is the essence of evolution, the substitution rate is at the same time also the *evolutionary rate*. And since different genes accumulate substitutions at different rates, the substitution rate is an important parameter of evolutionary change.

The spreading of a substitution from the moment of its occurrence in a single individual to its fixation in the population can be driven by chance alone (*genetic drift*) or it can be effected by the combined influence of drift and selection. When selection is involved, it means that an advantage (*positive selection*) or disadvantage (*negative selection*) is associated with possessing the particular substitution. (We mention negative selection for completeness although it does not come into consideration in our example, where the substitution has been fixed.) A substitution whose fate is determined by drift alone can be considered as *neutral*. To simplify somewhat the true situation, all synonymous substitutions are neutral because they do not change the phenotype, which is the target of selection. Nonsynonymous substitutions can, theoretically, be neutral, advantageous, or disadvantageous (deleterious).

In a gene, synonymous and nonsynonymous sites mutate with the same probability. Hence, if there is no selection, synonymous and nonsynonymous substitutions will occur at equal frequencies: the proportion $d_N/d_S = \gamma$ will equal 1. If negative selection tends to eliminate most of the nonsynonymous substitutions, the value of γ will be less than 1. Finally, if positive selection favors the nonsynonymous substitutions, the value of γ will be more than 1. The proportion γ thus provides a simple means of determining whether positive, negative, or no selection occurs at a gene. Note, however, that if substitutions are positively selected at half the sites and negatively selected at the other half, the gene will appear as selectively neutral when all the sites are lumped together. Only by knowing which sites are likely targets of positive selection and which of negative selection, and by treating these separately, can the presence of positive selection be inferred. Much of the earlier controversy about the effect of selection on *Mhc* genes can now be attributed to a failure to differentiate between sites influenced by positive and negative selection.

A plot of the number of synonymous or nonsynonymous substitutions against the time of gene divergence is often a straight line, which indicates that both types of substitution accumulate linearly with time, with the

regularity of a timepiece. This molecular clock is, however, ticking at a different rate for the synonymous and nonsynonymous substitutions, as well as for the nonsynonymous substitutions in genes at different loci.

BACK TO THE FUTURE: GENEALOGY OF *MHC* ALLELES

The replication of a gene produces two daughter copies; if these replicate again, four copies ensue, which give rise to eight copies in the next round of replication, which give rise to 16 copies, and so on. Of course, not all the copies survive; those that do not—either because the germ cells or the individuals that bear them failed to reproduce—terminate a branch of a dichotomously growing tree. If we could keep a record of who begot whom, it would not be unlike a genealogical tree constructed by a historian to show the lines of descent of a family.

Now, consider two genes that may or may not differ in their nucleotide sequence. If we were to follow their lines of descent back in time, we would eventually come to a point where the lines coalesce in a single ancestral gene. We can also reverse the process mentally by starting from the ancestor and following the divergence of the lines of descent toward the present. We can thus move back in time and then back to the future again at will. The process of coalescence and divergence can be interpreted mathematically or simulated by computer, and important generalizations concerning the behavior of genes in a population can be obtained (3, 4).

One simple question that can be answered by this approach concerns the mean number of generations one needs to go back to reach the point of coalescence for the two genes. Mathematical considerations show that this number is $2N_e$, where N_e is the *effective population size* (i.e. the number of breeding individuals in a randomly mating population; see Crow & Kimura—5). Hence, if we take 10,000 individuals as the mean effective population size and the mean generation time of 20 years, we can expect that most neutral alleles diverged $2 \times 10{,}000 \times 20 = 400{,}000$ years ago.

Since the average lifespan of a mammalian species is 2 my (6), most of the neutral polymorphisms found in the extant populations must have been generated after speciation. In younger species or in older species with large effective population sizes, some neutral alleles might have diverged before the species divergence, but on the whole, most of the neutral variability presumably postdates speciation. Moreover, because most of the polymorphism is believed to be nearly neutral (7), much of the polymorphism in general must be of recent origin. There are, however, genetic systems that do not behave according to the theory, and among these the *Mhc* is one of the most illustrious.

There is now a considerable body of evidence supporting the notion that the great majority of *Mhc* alleles present in current populations at the functional loci originated long before the origin of the extant species. The hypothesis suggesting that *Mhc* polymorphism is passed from one species to the next along an evolutionary line (the *trans-species hypothesis*) was first invoked when it became obvious that the genetic distances between certain *Mhc* alleles were very large (8). Support for the hypothesis was first obtained from the comparison of tryptic peptide maps of Mhc molecules isolated from different species of the house mouse (9). The identity of the maps of serologically indistinguishable allomorphs indicated that, contrary to the belief current at that time, the *Mhc* genes were not evolving rapidly. The observation of trans-specific sharing of serologically defined antigenic determinants (10) and of restriction enzyme sites (11) supported this conclusion. More convincing evidence was provided by the comparison of nucleotide sequences between genes isolated from different species (12–15; and many others). In primates, the mammalian order studied most extensively in this regard, sequence comparisons indicate that several of the allelic lineages were established before the divergence of apes and Old World monkeys more than 23 my ago (16–20). Distance estimates suggest that at least some of the lineages could be up to 40 my old. Taking 2 my as the average lifespan of a mammalian species, we can calculate that an allelic lineage this old must have passed through some 20 speciation events in the line leading to *Homo sapiens* and through many more such events in the lines extending to the species with which humans shared ancestors 40 my ago. The *Mhc* polymorphism has thus been passed on from species to species like a baton in a relay race.

Here is how the evolution may transpire in an idealized situation. Consider a particular *Mhc* locus and a population initially monomorphic at this locus. Assume a mutation has occurred at the locus in one of the individuals. In a population consisting of 10,000 breeding individuals and hence of 20,000 genes, the odds against the mutant allele "making it" (i.e. not being lost by random genetic drift) are 1:19,999. Consider that the new allele has succeeded despite the long odds against this happening and that it is increasing in frequency. In contrast to a neutral mutation in a standard gene, which under these circumstances would spread through the population until it replaced all the old alleles, the *Mhc* mutation may never reach the fixation point. Instead, its progress may be halted at a certain intermediate frequency, and the allele may either remain at this frequency or fluctuate over time, but neither disappear from the population nor replace the old allele. The mutation remains in a state balanced against loss or fixation, a state of *balancing polymorphism*, and it may remain so for millions of years. We discuss the reasons for this later.

While the new allele stabilizes its balancing act, another "lucky" mutation (one among myriads of "unlucky" ones) may begin to rise in frequency. Like the first mutation, it too establishes itself at a certain frequency between 0 and 1. After a long while, the idealized population may consist of a large number of alleles, all occurring at approximately the same frequency. (If we define polymorphism as a state in which an allele has the frequency of 1% or more, there cannot theoretically be more than 100 polymorphic alleles in a population at a given locus.)

We have thus far assumed that new alleles arise by mutations in genes that have not yet mutated (the "old" allele). But at some point "lucky" mutations will also begin to appear in genes that have mutated once already. An allele with two mutations may now begin to replace the allele with one mutation, and the same process may gradually supplant twice-hit genes for all the once-hit genes in the population. Subsequently, thrice-hit genes supersede twice-hit alleles, and so on, so that the alleles diverge further and further from each other. Since the "first generation" mutations do not all occur at the same time, and the same is true for all the subsequent "generations," the divergence of alleles will not progress in quite so orderly a manner as described. Rather, allelic lineages will form, each lineage consisting of a series of alleles differing by a different number of substitutions, but all more closely related to one another than to members of another lineage. The spectrum of allelic variability thus becomes discontinuous, broken into separate, more or less unambiguously delineated clusters. Hence, instead of evolving by fixation, as do most other genes, the *Mhc* genes evolve by *incorporation* of mutations into emerging allelic lineages. (This is not to say that evolution by fixation does not take place in *Mhc* genes; it does, but it is often obscured by the more strongly pronounced evolution by incorporation; see Klein & Takahata—21.)

While all this is going on, the population may divide into two or more noncommunicating daughter populations which may then evolve into new species. The founding populations of the new species are large enough so as not to lose the accumulated *Mhc* polymorphism. (There is not much evidence for the popular notion that new species arise from single pairs of individuals stranded in an isolated territory; many species are founded by populations of considerable size; see Klein et al—22.) The new species therefore take over the polymorphism accrued by the parental population and then make their own contribution to it. Now, however, there is no possibility of exchange between the two populations, and so the polymorphisms evolve separately in each species, as reflected in the genealogy of the alleles. As the populations evolve through successive speciations, the allelic lineages become superimposed on the species divergence so that when extant species are compared, the degree to which they share *Mhc*

polymorphism depends on the degree of evolutionary relatedness among them.

THE EVIDENCE FOR NATURAL SELECTION

Why do *Mhc* genes evolve differently from most other genes? If the evolutionary behavior of most standard genes is that expected under neutrality, then the different behavior of the *Mhc* genes must be attributed to the opposite of neutrality—natural selection, specifically, selection favoring balanced polymorphism. Indeed, there are arguments in support of balancing selection taking place at the functional *Mhc* loci. The most persuasive of these is based on the recent elucidation of the three-dimensional structure of Mhc class I molecules by Bjorkman et al (23, 24) and the prediction of the tertiary structure of class II molecules by Brown et al (25).

The Mhc molecules are now known to contain a highly specialized groove occupied by short peptides, which are acquired from the cytoplasm at the time the Mhc heterodimers assemble from their component polypeptide chains. The peptides are held in the groove by noncovalent interactions with amino acid residues constituting the peptide-binding region (PBR) of the Mhc molecules. The PBR residues of class I molecules reside in the $\alpha 1$ and $\alpha 2$ domains (encoded in exons 2 and 3, respectively), and those of the class II molecules in the $\beta 1$ and $\alpha 1$ domains (encoded in exon 2). Since the primary function of the Mhc molecules is binding peptides, the molecules can be divided functionally into two parts, PBR and non-PBR, which consist of approximately 57 and 280 (class I) or 16 and 220 (class II) residues, respectively. The ratios of nonsynonymous to synonymous substitutions (γ) calculated separately for the PBR and non-PBR reveal that these two parts evolve differently (26–29; see Table 1). In the PBR of the functional *Mhc* genes, the rate of nonsynonymous substitutions at nonsynonymous sites is significantly higher than that of synonymous substitutions at synonymous sites (γ values of up to 7, see Table 1), whereas the opposite is true for the non-PBR (γ values as low as 0.3; Table 1). Hughes & Nei (26, 27) have interpreted these observations as evidence that balancing (positive) selection favors amino acid replacements at the PBR positions and that purifying (negative) selection eliminates most amino acid replacements in the non-PBR part, presumably to retain the integrity of the Mhc molecules. In terms of selection, therefore, three types of sites can be recognized in the sequences coding for the functional Mhc molecules: positively selected sites (nonsynonymous sites of the PBR-specifying codons), negatively selected sites (nonsynonymous sites of codons specifying the non-PBR tracts), and neutral sites (all synonymous

Table 1 The ratio of the nonsynonymous to the synonymous substitution rate (γ) at the *HLA-A*, *-B*, *-DRB1*, and *-DQB1* loci

	HLA-A		*HLA-B*		*HLA-DRB1*		*HLA-DQB1*		*Mhc-SRB6**	
Parameter	PBR	Non-PBR	PBR	Non-PBR	PBR	Non-PBR	PBR	Non-PBR	PBR	Non-PBR
γ	2.7	0.4	3.5	0.4	4.8	0.3	4.0	0.4	0.2	0.3
$\hat{\gamma}$	4.5		6.8		7.5		5.6		—	
L_S	256.8		260.8		128.7		148.3		39.3	
L_N	691.8		684.5		399.5		427.2		127.7	
L_B	134.4		134.7		38.9		36.5		25.0	

γ was calculated as a d_N/d_S ratio, where d_N and d_S are nonsynonymous and synonymous distances (rates), respectively.

$\hat{\gamma}$ was calculated from the formula $\hat{\gamma} = \sqrt{2S/n^2}$, where $S = 2N_e s$, s is the selection coefficient, n is the number of alleles, and N_e is the effective population size (29). L_S, L_N, and L_B are the total number of synonymous, non-PBR nonsynonymous, and PBR nonsynonymous sites, respectively.

* Because only four *HLA-DRB6* alleles are known, we included in this case also *DRB6* alleles of chimpanzee, gorilla, orangutan, drill, and rhesus macaque.

sites). In *Mhc* pseudogenes, all sites should behave as neutral (γ values close to 1); the fact that this is often not the case (e.g. *DRB*6 in Table 1) is puzzling. One possibility is that the gene was functional in the past, became inactivated recently, and there has not yet been enough time to obliterate the earlier effects of selection. This may, however, not be the full explanation, and the *Mhc* pseudogenes will require further attention.

Another argument in favor of balancing selection affecting Mhc molecules is based on the Ewens-Watterson test (30, 31), which requires an explanation. Consider selectively neutral mutations occurring at a particular locus with the regularity and unpredictability of radioactive decay (21). Make the simplifying assumptions that the mutations do not repeat themselves, that is, each mutation occurs at a site at which no previous mutations occurred, so that all alleles generated by the mutations are different from one another (the *infinite alleles model of mutation*; see 32), and that the number of mutations present in the population steadily increases. The continuous influx of mutations into the population (*mutation pressure*) is opposed by genetic drift, which eliminates some of the mutations at random. At a certain stage in a finite population, the two opposing forces will balance each other out so that the number of new mutations infusing into the population by mutation pressure will equal the number of old mutations lost by random genetic drift. At this steady state, the number of neutral mutations (alleles) present in the population will remain constant, even though the composition of the mutations changes continually. The number of alleles present at the steady state will depend on the size of the population (N_e) and on the mutation rate (v): the larger the population size and the mutation rate, the larger the expected number of alleles. Genetic theory predicts that the expected number will depend on θ which equals $4N_ev$.

Consider a steady-state population of a certain size in which genes mutate at a certain rate. If we choose an individual randomly from such a population, there will be a certain probability, F, that this individual will carry a pair of identical alleles (i.e. it will be homozygous for these alleles; put differently, the alleles descended from the same ancestral gene that suffered a particular mutation). Intuitively, we would expect this probability to depend on the number of different alleles present in the population: the more alleles in the population, the lower the probability that we will find an individual with identical alleles. At the extremes, if all the genes were different, the probability of finding an individual with identical alleles would be 0; if all were the same, the probability would be 1 (certainty). In reality, the probability is somewhere between 0 and 1, and we can calculate it for different values of θ. The curve in Figure 1 shows the relationship between the expected number of alleles and the expected gene identity (F, homozygosity) for different values of θ.

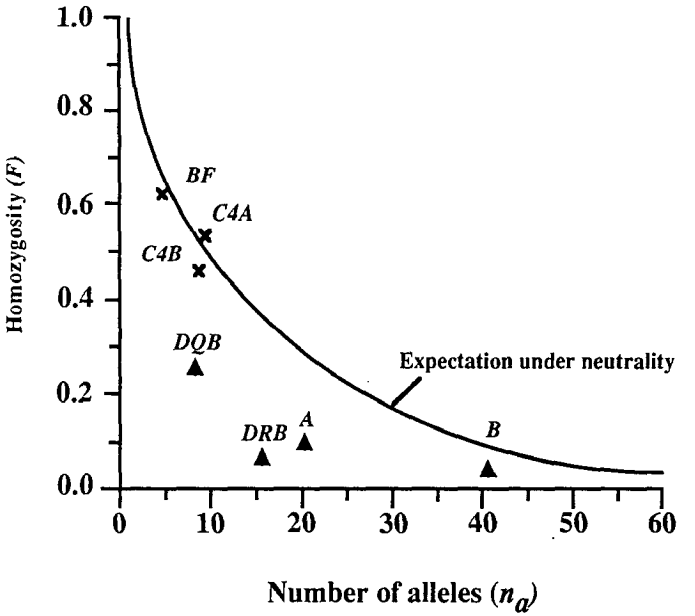

Figure 1 Homozygosity of *HLA* and complement (*C4*, *BF*) loci calculated from the gene frequencies reported by the Eleventh International Histocompatibility Testing Workshop, Yokohama, 1991. Homozygosity expected under neutrality is shown by the curve.

The assumption underlying these calculations is that the alleles are neutral. If the alleles are under selection, the curve shifts away from that expected under neutrality. Thus, by comparing a set of data with those expected under neutrality, we can assess whether there is selection on the products of a particular locus. All we need to do is plot the observed number of alleles at a particular locus against F. One of the principal laws of population genetics states that the frequency of homozygotes for a particular allele in a randomly mating population is p^2, where p is the frequency of this allele. In a population containing n alleles at frequencies $p_1, p_2, p_3, \ldots, p_n$, the homozygosity ($F$) is

$$\sum p_i^2 = p_1^2 + p_2^2 + p_3^2 + \cdots + p_n^2.$$

In other words, the value of F can be obtained by summing up the squared frequencies of all the alleles occurring in a population at a given locus.

When the Ewens-Watterson test is applied to the *Mhc* (the human *HLA* complex), departures from the values of homozygosity expected under neutrality are observed (33; and others; see Figure 1). The homozygosities of both class I and class II genes are consistently lower than those predicted under neutrality, while the homozygosities of the two closely linked com-

plement loci (*C4*, *BF*), for example, do not deviate significantly from neutrality expectations. The lower-than-expected frequency of homozygotes suggests an effect of balancing selection on the functional *HLA* genes. The Ewens-Watterson test has several drawbacks, however, which include large mean square error of the estimator for θ, low statistical power, and a requirement for populations in equilibrium (34).

The calculations underlying the Ewens-Watterson test have been based on data from global human populations. Deficiency of homozygotes has, however, also been documented for human isolates such as Saharan Tuareg (35) and Indian tribes of the lower Amazon (36), as well as for mouse experimental (37) and natural (38) populations. The deficiency is even more striking when one considers that because of their relative isolation, these populations have been undergoing a certain degree of inbreeding and hence breeding toward homozygosity.

Further support for an effect of selection is provided by the observation that certain *Mhc* alleles or haplotypes are associated with protection against Marek's disease in the domestic fowl (39, 40) and malaria in humans (41). The observation that the *HLA* class I gene frequencies show opposite but symmetrical north-south clines in the northern and southern hemispheres has been interpreted as supporting selection associated with climatic factors (42).

INTENSITY OF NATURAL SELECTION

A measure of selection intensity is *fitness*, the relative survival and reproductive success (contribution to future generations) of an individual or genotype. How important, then, are *Mhc* genes for the survival of an individual? There are two models that provide information pertinent to this question. The first are mice in which the expression of either class I or class II genes has been abolished by genetic manipulation (homologous recombination with defective genes: see 43–46). Both the class I– and class II–negative mice are not only viable, but they also survive under standard, clean animal house conditions. They may be susceptible to some virus infections but apparently cope as well as wild-type mice with other virus infections. The second model is provided by patients with the bare lymphocyte syndrome (BLS), believed to be caused by a defect in genes coding for factors that bind to the promoter regions of the class I and class II *Mhc* genes (47–50). Although the *Mhc* genes themselves are intact in BLS individuals, they are not transcribed and no Mhc molecules appear on the cell surface. The severity of the disease varies depending on the particular defect, but most children with BLS die before reaching the age of five years

and very few survive beyond 12 years of age. Thus, lack of Mhc molecules seems to have a more drastic effect on survival in humans than in mice. Clearly, however, the *Mhc* genes are not involved in any housekeeping function that would make their absence of expression absolutely incompatible with survival. Also, the two classes of *Mhc* genes apparently back each other up functionally to a large degree: when functions normally executed by one class fail, they may be taken over to some extent by the other class.

It is customary to assign the fitness value $w = 1$ to the genotype with the highest survival rate and highest reproductive success. Compared with the fittest genotype, the fitness of the other genotype (or genotypes) in the population is lower by a factor referred to as the *selection coefficient*, s, so that its fitness value is given by the expression $w = 1 - s$. Hence the selection coefficient is a quantitative measure of the reduction in fitness of a genotype in comparison to the fittest genotype, a measure of a selective disadvantage ($s = 1 - w$). There are various methods of determining the selection coefficient (51), none of them easy. We have attempted to estimate the selection coefficient of the *Mhc* genes by using some of these methods (52; Table 2), but such estimates are possible only under certain simplifying assumptions. We assume that all the *Mhc* alleles occur in the population at approximately equal frequencies, that heterozygotes have higher fitness than homozygotes, that the mutation rate is $u = 3.18 \times 10^{-6}$ per gene (nonsynonymous PBR sites) per generation (how this figure has been

Table 2 Estimated values of selection coefficient (s) for *Mhc* loci

s	Method	Reference
0.001–0.007	$2F^2S = \ln\left\{\frac{S}{16\pi M^2}\right\}^{a}$	Satta et al (52)
0.004–0.021	$2F^2S = \ln\left\{\frac{S}{16\pi M^2}\right\}^{b}$	Satta et al (52)
0.1 or less	Computer simulation	Klein et al (22)
0.028	Natural selection by malaria	Hill et al (41)

[a] Calculated from frequencies of alleles.
[b] Calculated from the number of alleles. For both calculations, the assumed effective population size was 10^5.

reached is explained later), and that the human effective population size has been, until recently, between 10^4 and 10^5 individuals (22, 53). The first estimate of the selection coefficient is based on a formula derived by Kimura & Crow (32). They came to the conclusion that given a mutation rate u, selection coefficient s, and the effective population size N_e, the number of polymorphic (common) alleles, n, which can be maintained at a locus in a finite population at equilibrium is $n = 1/F$ and that F is given by the formula

$$F \approx \sqrt{\frac{1}{2S} \ln \left\{ \frac{S}{16\pi M^2} \right\}}$$

where $S = 2N_e s$ and $M = N_e u$. We have turned the argument around and calculated the s from F. Since F has two meanings, first as the probability that a randomly chosen individual will be homozygous at a locus and second as the proportion of homozygous loci, it can be estimated either from the number or from the frequency of *Mhc* alleles (Table 2).

A second method of estimating the selection coefficient is based on computer simulation (22, 54) designed to mimic, in a simplified manner, the evolution of *Mhc* polymorphism in a population, given certain basic parameters such as population size and mutation rate. Assuming that the selection coefficient is the only unknown parameter, it can be estimated as the value required to maintain the observed degree of *Mhc* polymorphism in the population (Table 2).

Finally, a third method of estimating the selection coefficient is based on the effectiveness of protection provided by certain *HLA* alleles against *Plasmodium falciparum*, the causative agent of malaria (55). This estimate may, however, be the least realistic of them all because the observations on which it is based are subject to alternative interpretations.

All the estimates, however, suggest that the selection coefficient is relatively low, probably less than 0.02 (Table 2). In very simplistic terms, this means that fewer than 2% of *Mhc* homozygotes die or are otherwise disadvantaged in comparison with heterozygotes. This low value in turn, means that it will be very difficult to demonstrate selection directly in an experimental setting. For example, to show convincingly a selection-effected change in gene frequency from one generation to the next, more than 5000 progeny will have to be tested.

CAUSE OF NATURAL SELECTION

Selection is a process (51) that has a cause, an agent responsible for it. What is the cause of selection at *Mhc* loci? There are few experimental

data directly pertinent to this question, but there is a powerful logical argument based on indirect evidence: the only known primary function of Mhc molecules is presentation of peptides to T lymphocytes, which provide protection against parasites. Selection occurs at regions of the *Mhc* involved in the binding of these peptides; hence, the selection must be driven by parasites.

This conclusion has the universality that one would expect from a system apparently possessed by all vertebrates. The *Mhc* has thus far been demonstrated in cartilaginous fishes (56, 57), teleost fishes (58), amphibians (59, 60), reptiles (61), birds (62), and mammals (63). It is also expected to be found in agnathan fishes. The omnipresence of parasites needs no documentation. Hence, parasites can be the cause of selection at *Mhc* in all vertebrate classes. This generality is lacking from all the other causative agents that have been proposed as providing the selection pressure at the *Mhc*. *Mhc*-influenced mating preference (37) based on olfactory signals (64) may work well for mice (although even here its significance in wild populations has not been demonstrated unambiguously), but if it is real, it does not apply to the overwhelming majority of other vertebrates. It has been known since Darwin (65) that most females choose males not on the basis of their odor, but on the basis of courtship displays, song, coloration, and morphological features (66). Often, however, females don't choose males at all; males battle for access to females. These battles are decided by size and physical strength and not by smell. Hence, if female mice do indeed choose their mates according to *Mhc*-associated odors, the phenomenon could not be much more than a quirk of nature, without general significance. It is possible that *Mhc* genes contribute to the smell of mouse urine, but they cannot code for the horns of the sheep, the comb and wattle of a red jungle fowl, the plumage of a bird of paradise, and all the other secondary sexual characters used by males to court females. One could argue that the genes coding for these characters are linked to the *Mhc* as part of the strategy to advertise that the males are resistant to parasites [the Hamilton-Zuk (67) hypothesis]; but even this seems far-fetched. The argument requires that closely related taxons have very different genes closely linked to the *Mhc*, an extremely unlikely proposition. Similarly, since over 86% of vertebrates fertilize their eggs and develop their fetuses outside the mothers' bodies, fetal-maternal interactions (68) cannot be driving the evolution of the *Mhc*. Parasites must have been the primary agent of *Mhc* selection throughout the evolution of vertebrates.

If parasites are the driving force of *Mhc* evolution, why has it been so difficult to demonstrate associations between infectious diseases and *Mhc* polymorphisms? There may be several reasons. First, since the selection coefficients are so low, very large population samples would have to be tested to establish relationships between a particular infectious disease and

a particular *Mhc* allele; such samples have not been examined in any of the studies reported thus far. Second, the associations can be expected to be highly complex and hence difficult to disentangle. Each Mhc molecule must protect not against one, but against hundreds of parasites—different species and variants of the same species. Its capability to do this is demonstrated by the existence of *Mhc* monomorphic populations and species (69). It may therefore be somewhat naive to look for resistance alleles without taking into account this complexity of the host-parasite relationships. Third, virulent parasites effecting widespread infections may not be appropriate for revealing *Mhc*-parasite relationships. They represent situations in which the immune system either has failed or has been overridden temporarily by the agressivity of the parasite. The involvement of the *Mhc* may therefore be masked in such situations. It may be more rewarding to study parasites living in harmony with their hosts, for in such cases the *Mhc* could be responsible for coadaptation from the host's side. Fourth, the tremendous increase in the size of the human population in the last 7000 years and the dramatic change in the population structure may have obscured adaptive relationships between the human host and the parasites, relationships that may have coevolved for millions of years. It may therefore be more informative to study *HLA*-infectious disease associations in the remnants of the primitive human populations, in which the original population structure is preserved to some extent at least. To understand the mechanism of selection at *Mhc* loci, population subdivision should probably be taken into account.

The cause of selection must be distinguished from the mechanism (immunologists often confuse the two). Two mechanisms are currently considered as most likely to maintain *Mhc* variants in a population, overdominance and frequency-dependent selection (54). *Overdominance* is a condition in which the heterozygote manifests a trait more extremely than does either homozygote. In the context of selection, overdominance (*heterozygous advantage*) refers to a situation in which the fitness of the heterozygote is greater than that of either homozygote. The term *frequency-dependent selection* has many meanings; the one usually used in the context of the *Mhc* refers to a situation in which an allele (a particular phenotype) is at a relative selective advantage when rare; as its frequency increases, its selective advantage wanes. No experimental data are available to enable one to choose unambiguously between these two mechanisms.

SUBSTITUTION AND MUTATION RATES

The standard method of calculating substitution rates is based on the assumption that genes of two species (e.g. human and chimpanzee)

diverged after the species formed. This assumption, however, is invalid in the case of the *Mhc* genes, many of which began to differentiate from each other long before the divergence of the species. The application of the standard method to *Mhc* genes of closely related species would therefore result in a gross overestimation of the true substitution rate. One could avoid this problem by comparing the sequences of species such as human and mouse that diverged before the divergence of even the oldest alleles. This comparison would, however, lead to an underestimation of the true rate because many sites that suffered reverse mutations would appear as undiverged while others would be counted as having mutated once, although in reality they suffered multiple hits.

To avoid both these problems, Satta and her colleagues (70) designed a method that takes the trans-species evolution of *Mhc* alleles into account, identifies the gene pairs that diverged at about the same time as the species, and uses the distances between them for the calculation of substitution rates. The method requires that most of the alleles at a given locus have been sequenced in related species, a situation realized in the hominoid primates. If one compares each sequence with all other sequences in such a representative sample, one can expect the distances between genes to be a random variable reflecting the different divergence times of the alleles. The genes with the shortest distance should then be those that began to diverge at the time of the species divergence. Since the species divergence time is known, the rate of divergence of the *Mhc* genes can be calculated.

The application of this method to the primate *Mhc* sequences led to two surprising conclusions—that *Mhc* genes evolve with the regularity of a clock and that the synonymous substitution rate of the *Mhc* genes is among the lowest of all the genes for which substitution rates have been calculated. The clock-like regularity of *Mhc* evolution is illustrated in Figure 2, which shows a linear relationship between genetic distances and geological time. The linearity holds for the last 30 my, beyond which time the curve is artificially lowered by too many multiple hits difficult to correct by statistical means. It is observed not only for synonymous, but also for nonsynonymous substitutions, including those in the PBR. The constancy of the PBR nonsynonymous substitution rate means either that selection at Mhc molecules is relatively constant or that the changes in the selection coefficient (s) are correlated with changes in the effective population size (N_e) so that the product $N_e s$ remains the same (71).

The average nonsynonymous substitution rate for the PBR of the *Mhc* genes is 5.9×10^{-9} per site per year. Hence, contrary to expectations based on the fact that balancing selection plays such a prominent part in *Mhc* evolution, the nonsynonymous rate of these genes is unremarkable. It is similar to that of many other genes, including the non-*Mhc* "standard,"

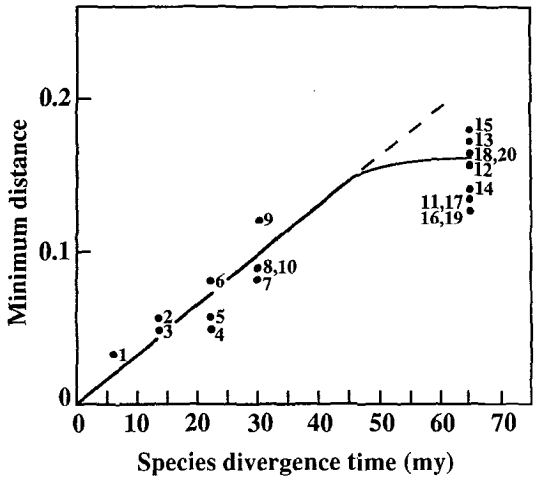

Figure 2 Constancy of the evolutionary rate of *DRB*1 genes. Each point represents the minimum distance between alleles in a given species pair, calculated from substitutions at synonymous sites. The pairs are these: 1, human vs chimp; 2, chimp vs orangutan; 3, human vs orangutan; 4, human vs macaque; 5, chimp vs macaque; 6, orangutan vs macaque; 7, human vs tamarin; 8, chimp vs tamarin; 9, macaque vs tamarin; 10, orangutan vs tamarin; 11, human vs cattle; 12, macaque vs cattle; 13, chimp vs cattle; 14, orangutan vs cattle; 15, tamarin vs cattle; 16, macaque vs dog; 17, human vs dog; 18, chimp vs dog; 19, orangutan vs dog; 20, tamarin vs dog; my, million years. (Modified from Satta et al (70)).

the globin genes. On the other hand, the synonymous substitution rate of the *Mhc* class II genes is remarkable in that it is so low. The average rate is 1.18×10^{-9} substitutions per site per year. Very few other primate genes evolve at such a low rate (72, 73; Figure 3). Low synonymous substitution rate at *Mhc* genes is also suggested by other studies (26, 27, 74). The implications from these figures are that it takes approximately 0.7 my to incorporate one substitution into a class I gene, and the probability is about equal that the substitution will occur in the PBR or in the rest of the gene. (Although the PBR is considerably shorter than the rest of the gene, positive selection raises the probability of substitution in this region compared to the non-PBR regions.) At the class II loci, it takes on average 1.6 my to incorporate one substitution into a gene, and the probability that the substitution will occur in the PBR is approximately two times lower than the probability that it will be in the rest of the gene.

The low substitution rate can be explained by the nucleotide composition of the *Mhc* genes. Wolfe and his colleagues (75) demonstrated that the variation in the synonymous substitution rate correlates with the frequency of guanine and cytosine (the G+C content) in the third codon position of

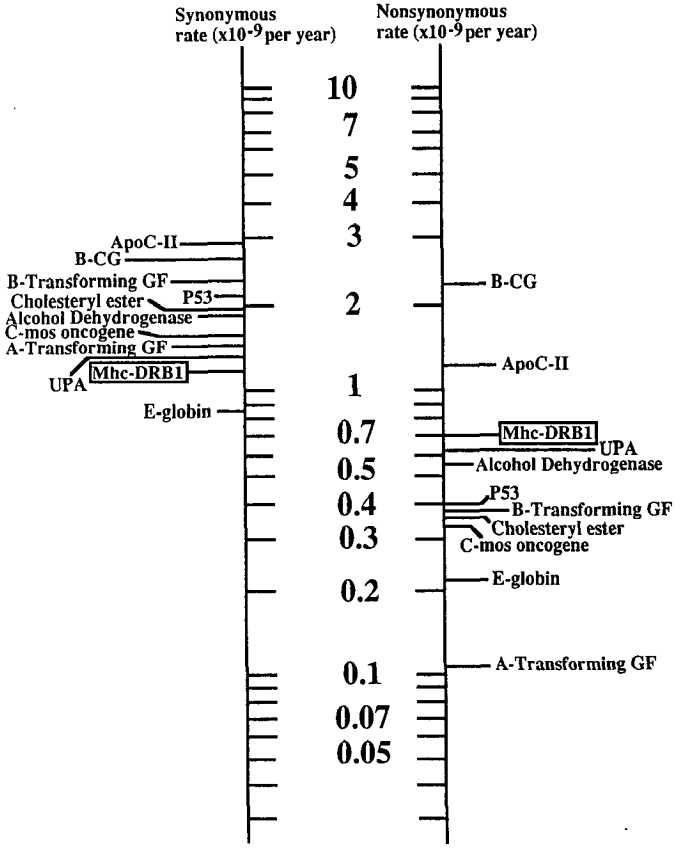

Figure 3 Synonymous and nonsynonymous substitution rates of primate *DRB* genes as compared to selected primate non-*Mhc* genes. The *DRB* rates are from Satta et al(70). References from the other DNA sequence data are available on request. The substitution rates of the non-*Mhc* genes were calculated by the method of Li et al (72), assuming divergence time of 25 my between *Homo sapiens* and Old World monkeys.

mammalian genes. They observed that genes with high (>55%) or low (<45%) G+C content have low synonymous substitution rates and that the synonymous rate is a good measure of the mutation rate. The basis for the correlation is not easy to comprehend, but one possibility is that the G+C content is determined by the composition of the nucleotide precursor pool during DNA replication and that the composition varies according to the time of replication of the gene in the cell cycle.

The nucleotide composition of the *Mhc* genes can be considered at two levels—at the isochore and at the gene level. Isochores are regions of the

mammalian chromosome with relatively homogeneous nucleotide composition and measuring from 200 kilobases (kb) to 1000 kb (76). The *HLA* complex straddles two isochores: the class II genes lie in the region of moderate G+C content, whereas the class I genes are located in a (G+C)–rich isochore, the border between the two isochores falling in the region between *HLA-DRA* and *C4* genes (77). At the gene level, however, the class II genes have a high G+C content (70%–80%; see 78), so that they fit the pattern observed by Wolfe and his colleagues for other mammalian genes.

The implication of the low substitution rate is that contrary to popular belief, the mutation rate of the *Mhc* genes must be low. According to population genetics theory (1, 2, 7), the synonymous substitution rate is more or less equal to the mutation rate. Assuming hominid generation time of 20 years (79), the substitution rate of 1.18×10^{-9} substitutions per synonymous site per year translates into a mutation rate of 6.1×10^{-6} per class I gene (synonymous sites only) per generation, and to 3.9×10^{-6} per class II gene (synonymous sites only) per generation. This estimate is about two orders of magnitude lower than that based on mutations actually detected at the class I *H*–2 loci in the mouse (80). If, however, most of the detected *H*–2 "mutations" are in fact recombinations ("gene conversions"), as is generally believed (81), they do not provide a true measure of the mutation rate. Although, taken very strictly, our calculations pertain only to the synonymous sites of the *Mhc*, there is no reason to believe that they do not apply to nonsynonymous sites as well. The estimate of 2.4×10^{-8} mutations per site per generation can therefore be extrapolated to encompass the entire *Mhc* gene. It then comes to 2.6×10^{-5} mutations per class I gene (all sites) per generation and to 1.7×10^{-5} mutations per class II gene (all sites) per generation. These values are comparable to the mutation rate of an average human [Table 5.8 in Vogel & Motulsky (82)] or mouse [Tables 2 and 3 in Klein (80)] gene.

EVOLUTION OF NON-PBR SITES

The balancing selection operating at the PBR sites is not without an effect on the rest of the *Mhc* gene. One of the consequences is that the synonymous sites in the non-PBR parts of the gene are more polymorphic than synonymous sites of a standard, non-*Mhc* gene. A measure of the accumulation of genetic variation in general is the amount of heterozygosity. If the combined frequency of homozygotes (F) and the heterozygotes (H) in a population is 1, then the frequency of heterozygotes is $H = 1 - F$. Since, as stated earlier, the frequency of homozygotes is given by the expression Σp_i^2, where p_i is the frequency of individual alleles, the

heterozygosity (H) is given by the expression $H = 1 - \Sigma p_i^2$. For example, if there are two alleles in a population at frequencies $p_1 = 0.5$ and $p_2 = 0.5$, then $H = 1 - [(0.5)^2 + (0.5)^2] = 0.5$; if there are three alleles at equal frequencies, $H = 1 - [+(0.33)^2 + (0.33)^2] = 0.67$. Hence, the amount of heterozygosity rises with the increasing number of alleles present in the population, but the increase depends not only on the number, but also on the frequency of the individual alleles (e.g. if there are two alleles at frequencies $p_1 = 0.75$ and $p_2 = 0.25$, then H is only 0.37). Heterozygosity can be calculated for the entire gene (protein), for individual codons (amino acid positions), or individual nucleotide sites. It can have slightly different meanings accordingly. A heterozygosity of 0.42 per gene means that 42% of individuals in a population are heterozygotes.

Hedrick and his coworkers (83) used this measure to demonstrate that the average heterozygosity at PBR amino acid positions is 6.7 times higher than that at the non-PBR positions in the class I HLA molecules (heterozygosity of 0.20 and 0.03, respectively). The observed non-PBR heterozygosity is, in turn, 10–15 times higher than the 0.002 to 0.004 value of the human insulin, β-globin, and growth hormone (2). A similar conclusion can also be reached by calculating heterozygosity at nucleotide sites (Table 3).

Table 3 Heterozygosity at nucleotide sites of the *HLA-B* and *HLA-DRB1* genes

Region	*HLA-B*		*HLA-DRB1*	
	h	$E(h)$	h	$E(h)$
PBR-nonsynonymous sites	0.119	0.219	0.299	0.390
Non-PBR-nonsynonymous sites	0.015	0.015	0.021	0.025
Entire gene-synonymous sites	0.037	0.038	0.058	0.073

Heterozygosity, h_{ij}, between two alleles, generally i and j, was calculated from the formula $h_{ij} = 2p_i p_j d_{ij}$, where p_i and p_j are frequencies of the i and j alleles and d_{ij} is the per site genetic distance between the two alleles. The overall heterozygosity (h) was then obtained by summing up the individual h_{ij}:

$$h = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} h_{ij}$$

where n is the number of alleles in a population (21 for *HLA-B*, 13 for *DRB1*). The following alleles (frequencies) were included in the analysis: *HLA-B*0702* (0.112), **0801* (0.065), **1301* (0.045), **1402* (0.039), **1801* (0.047), **2702 (0.041)*, **3501* (0.101), **3701* (0.030), **4001* (0.055), **4002* (0.004), **4101* (0.019), **4201* (0.019), **4401* (0.104), **4601* (0.029), **4701* (0.011), **4901* (0.019), **5101* (0.085), **5201* (0.070), **5301* (0.018), **5701* (0.032), **5801* (0.055). *HLA-DRB1*0101* (0.0903), **1501* (0.1374), **1601* (0.0321), **0404* (0.1747), **0301* (0.0157), **0302* (0.0092), **1103* (0.0923), **1201* (0.0582), **1301* (0.0949), **0701* (0.1001), **0802* (0.0864), **0901* (0.0805), **1001* (0.0281). The frequencies were those reported at the Eleventh International Histocompatibility Testing Workshop, Yokohama 1991.

Expected heterozygosity $E(h)$, was computed using the formula $E(h) = 2\alpha\mu\gamma/(1 + 2\alpha\mu\gamma)$; for the synonymous sites $\gamma = 1$: for non-PBR-nonsynonymous sites and PBR nonsynonymous sites γ values are given in Table 1.

According to population genetics theory and under certain simplifying assumptions, the expected heterozygosity in a population is: $E(h) = 2\alpha\mu/(1+2\alpha\mu)$ where ($\alpha = n^3/(2\sqrt{2uS})$, μ is the mutation rate per site per generation, u is the mutation rate per PBR-nonsynonymous sites per generation, n is the number of alleles in the population, and $S = 2N_e s$ (N_e is the effective population size and s is the selection coefficient). Applied to the *Mhc* genes, α is the time (in generations) during which n nonsynonymous substitutions accumulated at PBR sites, while $2\alpha v$ substitutions occurred at the synonymous sites, where v is the mutation rate per synonymous sites of a gene per generation (29). The expected heterozygosity values are in reasonably good agreement with the observed values (Table 3), and hence the assumptions underlying the theory must be realistic.

One might be tempted to ascribe the high level of polymorphism at the synonymous sites of the *Mhc* loci to a hitchhiking effect of selection at nonsynonymous PBR sites: As the frequency of an allele generated by a nonsynonymous substitution rises, driven by balancing selection, one could assume that synonymous substitutions will be dragged along simply because the sites are so closely linked to the PBR sites. Closer scrutiny, however, forces us to reject this argument. The substitution rate at nonsynonymous PBR sites (combined class I and class II genes) is approximately five times higher than that at the synonymous non-PBR sites (it is 5.9×10^{-9} per site per year at the former and 1.2×10^{-9} per site per year at the latter). Hence, it takes about 1 my to incorporate one nonsynonymous substitution and 5 my to incorporate one synonymous substitution. To be hitchhiked, a synonymous substitution would have to occur in the same gene simultaneously with a nonsynonymous PBR substitution, or at least in the stage when the frequency of the selected substitutions is on the rise. Because of the discrepant rates, the probability of this happening is very low.

The reason that *Mhc* genes differ by more synonymous substitutions than do non-*Mhc* genes is not hitchhiking but simply the long persistence time of discrete allelic lineages at the *Mhc* loci. While alleles at most non-*Mhc* loci have a persistence time shorter than the life-span of a species, allelic lineages at *Mhc* persist for many millions of years and thus provide sufficient time to accumulate large numbers of synonymous substitutions independently in each lineage, in spite of the low substitution rate.

Finally, a word about the non-PBR nonsynonymous sites in the *Mhc* genes. These sites are under negative (purifying) selection because of functional constraints on the molecule. The degree of overall functional constraint, γ, on the non-PBR nonsynonymous sites relative to the synonymous sites can be estimated from the formula:

$$\gamma = \left(\frac{A_N}{L_N}\right)\left(\frac{L_s}{A_s}\right)$$

where A_N is the mean number of non-PBR nonsynonymous substitutions per gene, A_s is the mean number of synonymous substitutions per gene, L_N is the total number of non-PBR nonsynonymous sites of the gene, and L_s is the total number of synonymous sites in the gene (78). For a nonfunctional gene, one expects $\gamma = 1$, whereas for a completely conserved gene (no acceptable nonsynonymous substitutions), $\gamma = 0$. The calculated γ values of both class I and class II functional *HLA* genes are close to 0.33 (78). For comparison, γ values of histone H4, insulin C-peptide, and interferon β 1 genes are 0.004, 0.201, and 0.467, respectively, the average being 0.189 (72). Hence, the average γ value of *HLA* genes is approximately twice as large as that of other genes, and this result indicates that the purifying selection taking place at the non-PBR nonsynonymous sites is not particularly strong.

The basic parameters characterizing the molecular evolution of the *Mhc* genes are summarized in Table 4.

CHANGING PERSPECTIVE

Since the true extent of variability at the *Mhc* loci was first realized, it became fashionable to think of the *Mhc* in the superlatives of an American TV commercial. The substitution and mutation rates were supposed to be the highest of any genetic system known, recombinations of all kinds rampant, allelic turnover very rapid, selection extremely strong. We have attempted to document that this view of the *Mhc* is false. Both the substitution and mutation rates of the *Mhc* genes are low, the high frequency

Table 4 Basic parameters characterizing the molecular evolution of the primate *Mhc* genes

Parameter	Value
Age of allelic lineages	>30 million years
Selection coefficient	<0.02
Nonsynonymous substitution rate at the PBR	5.9×10^{-9}/site/year
Synonymous substitution rate	1.2×10^{-9}/site/year
Average incorporation time of nonsynonymous substitutions at the PBR	1.3 my (class I) 4.5 my (class II)
Average incorporation time of synonymous substitutions	3.2 my (class I) 5.0 my (class II)
Mutation rate	1.7×10^{-5}/gene/generation (class II)
Functional constraint (non-PBR)	~0.33

of recombination remains undocumented (84), allelic turnover is slow, and both the positive and negative selection pressure is mild. This reappraisal does not make the *Mhc* less interesting by any means, but it does make a lot more sense in the evolutionary context. It also reemphasizes a point that many contemporary immunologists seem to be forgetting—that a biological system can only then be fully understood when its evolutionary history has been elucidated. The *bel canto* of evolutionary biology is contributing tremendously toward this elucidation by permitting us to examine aspects of the *Mhc* that might be inaccessible experimentally. It is thus adding lustre to the *Mhc* studies at a time when their old aura seems to be fading.

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GENE THERAPY OF THE IMMUNE SYSTEM

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Abstract

Many applications of somatic gene therapy relate to the immune system. Several forms of inherited immunodeficiencies are candidates for treatment by gene transfer. Adenosine deaminase (ADA) deficiency causes a form of severe combined immunodeficiency. Stable gene transfer and expression of human ADA has now been obtained in hematopoietic stem cells of mice and, more recently, in large animals. The human ADA has also been introduced and expressed in the primitive human hematopoietic progenitor cells that initiate long-term bone marrow culture. Clinical trials of gene therapy for ADA deficiency have been initiated. The initial protocols were aimed at the correction of peripheral blood T lymphocytes, but recent strategies are attempting ADA gene transfer into peripheral blood or bone marrow stem cells. Other immunodeficiencies that may soon be amenable to somatic gene therapy include leukocyte adhesion deficiency and chronic granulomatous disease. Gene therapy may also be applied to the treatment of acquired disorders. In theory, the hematopoietic stem cells of a human immunodeficiency virus (HIV)-infected patient could be genetically modified and used to reconstitute an HIV-resistant hematopoietic system. Various strategies are currently being investigated to achieve this "intracellular immunization" against HIV. These include the transfer of genes encoding

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recombinant soluble CD4 molecules, suicide genes under the control of HIV-inducible promoter, and anti-HIV ribozymes. Gene transfer could also be used in the treatment of cancer to increase the immune response of the host, to activate prodrugs specifically in tumors, or to protect normal tissues against the toxicities of conventional treatment. Recent progress in all of these applications of gene therapy is reviewed here.

INTRODUCTION

Remarkable progress has marked the field of gene therapy during the last three years. Gene transfer experiments have been performed in humans and have confirmed that defective retrovirus vectors can be safely used to transfer new genetic information to the lympho-hematopoietic system. Experimental evidence also indicates that new genetic information can be efficiently introduced into primitive and long-lived hematopoietic stem cells not only in mice, but also in large outbred animals and most probably in humans. The genes affected in some of the most prevalent and serious hereditary disorders such as cystic fibrosis and Duchenne muscular dystrophy have been cloned, adding important new targets for gene therapy. Not only inherited diseases, but also relatively common acquired disorders such as cancer and infection by the human immunodeficiency virus (HIV) have become prime candidates for the gene therapy approach. New methods of gene transfer have been developed which greatly facilitate gene transfer in tissues that were previously less accessible. One manifestation of this progress is the growing number of approved human gene transfer trials. As of June 1992, nine gene marking and eight gene therapy experiments had been approved by the recombinant DNA Advisory Committee (RAC) in the United States, while another gene marking experiment and three gene therapy trials have been approved in France, Italy, China and the Netherlands (1, 2). Thus, the era of human gene therapy is already upon us. It is important to remember, however, that only somatic gene supplementation rather than gene repair is currently feasible. Thus, inasmuch as inherited disorders are concerned, only recessive traits are amenable to this form of treatment. Here, we review recent progress in somatic gene therapy as it applies to disorders affecting the immune system. In doing so, we try to indicate which goals have been attained, as well as the areas that will require further development.

GENE TRANSFER VECTORS

Although other methods of gene transfer such as adenovirus-based vectors (3) or direct injection of DNA (4) are being perfected and will almost

certainly play an important role in gene therapy of disorders affecting the lung, liver, or muscle, recombinant defective retroviruses remain the vectors of choice for the lympho-hematopoietic system (5–8). Defective retrovirus vectors, which are derived from disabled mouse RNA tumor viruses, are produced by replacing the structural genes of the provirus (the DNA form of the virus) with the genetic information to be transferred. To complement these defective proviruses, several "packaging" cell lines have been constructed, usually in mouse fibroblast cells. Packaging cell lines are made by stably transfecting the sequence for the structural genes of a retrovirus lacking its own "packaging signal" (the viral sequence required for packaging of the RNA into viral particles). Transfection of a defective retrovirus possessing an intact packaging signal into a packaging cell line results in the production of viral particles containing RNA copies of the recombinant vector. The resultant "defective" virus is able to infect target cells and, after reverse transcription into DNA, to become integrated into the host cell genome, thereby achieving gene transfer. However, because it lacks the virus structural genes, it is unable to replicate. In the most recent packaging cell lines, the virus structural genes have been separated between two independent plasmids, and additional deletions have been included to minimize the regions of overlap between the vector and the helper sequence of the packaging cells. This effectively prevents the generation of replication-competent virus through recombination events (9–12).

The primary advantages of retrovirus vectors are their efficiency of gene transfer and their expression in a wide range of cell types without gross deletion or rearrangement of the surrounding host DNA. Their main disadvantages are the size constraint (a maximum of 7 to 8 kb of added sequence), and the dependence on active DNA replication for efficient integration (13, 14). This limits the efficiency of gene transfer into cells that are predominantly quiescent and cannot be induced to proliferate. While the generation of replication-competent (helper) virus appears to be preventable and can be monitored appropriately, even defective retroviruses could in theory lead to malignant transformation by insertional mutagenesis. Such an event has not yet been observed in animals, nor in clinical trials in the absence of replication competent virus. It is therefore difficult to put an exact estimate on this risk. However, the recent report of thymic lymphoma developing in three of eight rhesus monkeys transplanted with bone marrow that was contaminated with replication-competent virus (15) underscores the need for stringent monitoring of replication-competent virus in human gene transfer experiments.

Although it is difficult to make generalizations regarding an optimal retrovirus vector construction, our experience as well as that of others is

that simplified vectors with a single transferred gene result in higher virus titers and higher levels of expression (16–19). These vectors also include a sequence 3′ to the classical ψ packaging signal, the so-called *gag*$^+$ sequence, which has been shown to increase the efficiency of viral RNA packaging (20). Figure 1 shows an example of such a vector which we have used successfully to transduce human adenosine deaminase (ADA) expression in mouse and human hematopoietic cells (21, 22). Finally, the use of the potent viral promoter/enhancer in the long terminal repeat (LTR) usually results in better expression than heterologous promoters because of transcriptional interference between competitive promoters. This promoter interference was overcome, however, when the virus enhancer was deleted from the 3′LTR of the vector (23) or when the gene and promoter were inserted outside the transcriptional unit of the vector in the 3′LTR (24).

IMMUNODEFICIENCIES AND DISORDERS OF PHAGOCYTIC CELLS

Several inherited immunodeficiencies and disorders of phagocytic cells that result in recurrent life-threatening infections, are candidates for somatic gene therapy. Table 1 lists a number of inherited immunodeficiencies, with the chromosomal location of their locus (25) and, where known, the gene involved. Here, we review recent progress made toward the somatic correction of some of these disorders.

Adenosine Deaminase Deficiency

BACKGROUND The inherited deficiency of adenosine deaminase (ADA) is a rare autosomal recessive disorder responsible for 15% to 25% of all cases of "severe combined immunodefiency" (SCID) (26). The pathophysiology of the disorder involves selective toxicity of nonmetabolized adenosine and 2′-deoxyadenosine, and accumulation of dATP in immature T and, to a lesser extent, B cells. It has been speculated that the accumu-

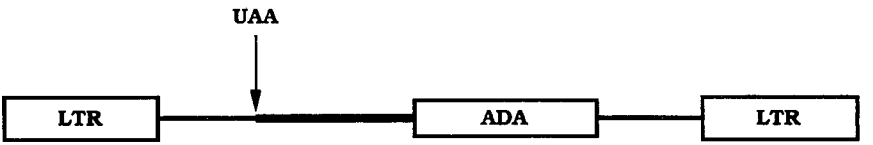

Figure 1 N2stADA retroviral vector. The heavy line indicates the *gag* sequence (nucleotides 564 to 1038 in Moloney-murine leukemia virus). LTR, long terminal repeat; UAA, translational stop codon replacing the natural state codon of the *gag* sequence to decrease the risk of replication-competent virus production; ADA, human ADA cDNA.

Table 1

Disorder	Chromosomal map location	Gene affected
Autosomal inheritance		
Congenital thymic aplasia (Di George syndrome)	22q11	nd
Selective IgA deficiency	nd	nd
Adenosine deaminase deficiency	20q13.11	adenosine deaminase
Purine phosphorylase deficiency	14q13.1	purine phosphorylase
Ataxia telangiectasia (complementation groups A and C)	11q22-23	nd
Common variable immunodefiency	nd	nd
Chronic mucocutaneous candidiasis	nd	nd
Hyper IgE immunodeficiency (Job syndrome)	nd	nd
Chronic granulomatous disease (autosomal recessive)		
—neutrophil cytosolic factor 1	7q11.23	p47-phox
—neutrophila cytosolic factor 2	1q25	p67-phox
—cytochrome b negative	16q24	p22-phox
X-linked inheritance		
X-linked chronic granulomatous disease	Xp21.1	gp91-phox
X-linked agammaglobulinemia	Xq21.3-q22	nd
X-linked lymphoproliferative disorder	Xq25	nd
Wiskott-Aldrich syndrome	Xp11.3-p11.2	nd
X-linked immunodeficiency with hyper IgM	Xq24-q27	nd
X-linked severe combined immunodeficiency	Xq13.1-q21.1	nd

lation of dATP alters the regulation of ribonucleotide reductase, a highly regulated enzyme required for cell replication. Alterations in DNA methylation pattern via inhibition of S-adenosyl homocysteine hydrolase have also been implicated. Although ADA deficiency is a rare disorder, it has for several reasons been regarded as an optimal model to develop somatic gene therapy (5–7). First, ADA deficiency is correctable via allogeneic bone marrow transplantation (BMT), i.e. by the introduction of genetically normal (or corrected) hematopoietic cells; however, only one third of patients have a suitably matched donor. Second, the alternative treatment with a stabilized form of bovine ADA linked to polyethylene glycol (PEG-ADA) can partially correct the defect, but this results in variable immune functions and can be complicated by the development of inhibiting antibodies. This raises concern regarding the long-term efficacy of PEG-ADA

for the prevention of life-threatening infections. Third, it is likely that unregulated or imperfectly regulated expression of the transferred gene (such as can be obtained with viral vectors) would nevertheless be beneficial to ADA deficient patients. Finally, an in vivo selection in favor of corrected cells is very likely to take place in the case of ADA deficiency, which is again an uncommon advantage. An in vivo selection in favor of ADA "normal" cells is evident from the outcome of allogeneic BMT given without preparative bone marrow ablation: in cases where this procedure is successful, the lymphoid system of the patient becomes of donor origin while the myeloid system continues to be derived from host stem cells (27).

GENE TRANSFER IN MOUSE HSC Initially, the hematopoietic stem cell (HSC) with long-term repopulating ability for all lymphoid and myeloid lineages was identified as the optimal target for gene therapy of ADA deficiency and other disorders affecting the lympho-hematopoietic system. Stem cells, however, constitute only approximately 0.01% of all nucleated bone marrow cells and are predominantly quiescent.

The efficiency of gene transfer into HSC is generally evaluated by analyzing the stability of expression of the transferred gene in several lympho-hematopoietic tissues for at least 4 to 6 months posttransplantation. Recently, gene transfer in mouse HSC has also been evaluated by analyzing the presence and expression of the transferred gene in the hematopoietic tissues of secondary recipient mice that were transplanted with bone marrow cells harvested from primary recipients. Early reports demonstrated successful transfer and long-term in vivo expression of selectable genes in mice following transplantation with transduced bone marrow cells (i.e. cells infected with replication-defective retrovirus vector) (28–30), but stable expression of disease-related genes was more difficult to obtain. Belmont et al (16) were the first to report successful long-term in vivo expression of transduced human ADA in mice. Although replication-competent retroviruses were present in those experiments, indirect evidence suggested that the long-term expression of human ADA resulted from efficient gene transfer into stem cells rather than from ongoing propagation of the vector in more mature cells. Several groups, including Belmont and colleagues, have since been able to demonstrate retroviral gene transfer of human ADA into mouse HSC and long-term in vivo expression of the transduced enzyme in the absence of replication competent retrovirus (21, 31–35). The levels of expression in different tissues varied between experiments, but stable and potentially therapeutic levels of expression of the human enzyme have been obtained in all lympho-hematopoietic tissues.

The factors that determined efficient retrovirus-mediated gene transfer into murine HSC were: (*a*) pre-treatment of the bone marrow donors

with 5-fluorouracil, which kills rapidly dividing hematopoietic cells, and indirectly increases the fraction of the HSC that will enter the cell cycle to replenish the hematopoietic system; (*b*) co-cultivation of the bone marrow cells with high-titre virus-producing cells (preferably with titers $\geqslant 10^6$ infective particles per ml); and (*c*) stimulation of the bone marrow cells before and during the exposure to the vector with hematopoietic growth factors that induce the proliferation but not the differentiation of HSC. Examples of growth factors that have been used successfully include a combination of interleukin–3 (IL–3) and IL–6, and leukemia inhibitory factor (36, 37). Purified murine hematopoietic stem cells were also efficiently transduced (38), suggesting the possibility that humans could eventually be transplanted with relatively small numbers of genetically modified and purified HSC. This would not only facilitate the procedure, but also decrease the risk of an insertional mutation leading to a malignancy.

GENE TRANSFER IN THE HEMATOPOIETIC SYSTEM OF LARGE OUTBRED ANIMALS

Several groups have attempted to obtain stable expression of human ADA or other transduced genes in large outbred animal models. It is felt that this would more closely reproduce the conditions of human gene therapy where a patient's own bone marrow cells would have to be subjected to gene transfer before an autologous BMT. Because of their phylogenetic proximity to humans, monkeys constitute particularly attractive animal models. Low levels of expression of human ADA and bacterial neomycin phosphotransferase (Neo^R) were obtained in the peripheral blood cells of cynomologous monkeys for up to 4 months following BMT with retrovirally transduced cells (39). Another group was able to detect vector sequence in the neutrophils of the peripheral blood for up to 3 months in rhesus monkeys that received bone marrow cells cocultivated with very high-titer vector producing cells (40). However, these cells also produced replication competent virus and a number of animals have since become viremic and developed thymic lymphoma (15). There might therefore have been persistent propagation of the vector due to the presence of helper virus rather than instead transfer into primitive cells. More recently, a third group has reported low level expression of human ADA for up to 1 year in rhesus monkeys following cocultivation of bone marrow cells with helper virus–free virus producing cells in the presence of rhesus monkey IL–3 (41).

Early experiments in dogs resulted in gene transfer only in committed, short-lived hematopoietic progenitors (42). More recent studies showed the persistence of a retroviral vector in 0.1 to 10% of canine progenitor cells from the bone marrow for up to 2 years in the absence of helper-virus

(43, 44). Intermittent expression of neomycin resistance was documented in one of these studies, but ADA expression was not detected (43). Both studies showed that supernatant infection while bone marrow cells were maintained in long-term bone marrow culture (LTC) was an efficient method of gene transfer. Drug-resistant hematopoietic progenitor cells were also identified in the bone marrow progenitor of some cats for more than 2 years after retroviral transduction and transplantation (45). However, the helper-contaminated vector previously used in monkeys was also used in this study, making it impossible to draw a firm conclusion regarding gene transfer into HSC. Finally, two groups have studied in utero retroviral gene transfer into fetal hematopoietic progenitor cells: 2 out of 9 sheep showed intermittent persistence of drug-resistance in 0 to 8.5% hematopoietic progenitor cells for 8 months to 2 years following in utero exchange transfusion with transduced cells (46); and vector sequence was detected by Southern analysis in the bone marrow of 3 out of 9 rats at 26 weeks following injection of viral vector into the liver at 16 days of gestation (47). However, the experiments involving sheep were also conducted with the high-titer vector contaminated with replication-competent virus.

In conclusion, while constant progress is being made toward achieving efficient retrovirus-mediated gene transfer into HSC of large outbred animals, the stable high level expression of transduced genes observed in various lympho-hematopoietic tissues of mice has not yet been obtained in these species. These results may partly reflect biological differences between mice and those species, for instance, a potentially lower density of receptors for the retrovirus on HSC of large animals (48). More probably they reflect two other factors: (*a*) that optimal conditions for the in vitro growth of hematopoietic cells have been less well characterized in these species than in the murine and human system, thus limiting the efficiency of retroviral gene transfer; and (*b*) that the optimal conditions to perform bone marrow transplantation are also less well defined in these species, often resulting in endogenous reconstitution and poor engraftment of the genetically modified cells (40, 48). More efficient retroviral gene transfer into HSC will predictably be obtained with improvements in these two aspects.

GENE TRANSFER IN HUMAN HSC A number of genes have been transferred and expressed into human hematopoietic cells following retrovirus-mediated gene transfer. These include the genes for neomycin phosphotransferase (49), a mutant form of dihydrofolate reductase (DHFR) conferring resistance to methotrexate (49), β-globin (50), ADA (51), and glucocerebrosidase (52). In those studies, only hematopoietic progenitor

cells that form colonies in vitro 10 to 14 days after gene transfer or cells that were maintained in liquid culture for short periods were analyzed. The LTC system offers the possibility of evaluating gene transfer into more primitive human hematopoietic progenitors (53). Preexisting clonogenic progenitor cells disappear (by death or differentiation) within 5 weeks of long-term culture. Most clonogenic cells that can be recovered after that time are derived from more primitive cells that were not in themselves clonogenic when the culture was initiated (54). These LTC-initiating cells may be functionally related to the primitive stem cells. The successful use of cells derived from human LTC for autologous bone marrow transplantation supports this view (55). Following cocultivation of human bone marrow cells with cells producing a Neo^R vector in the presence of hematopoietic growth factors (recombinant granulocyte/macrophage colony stimulating factor (GM-CSF) and IL–1β, or conditioned medium from a human stromal cell line), a stable proportion of approximately 30% of neomycin-resistant clonogenic progenitor cells could be derived from cells maintained in LTC for up to 6 weeks (56). We have used a combination of recombinant human IL–3 and IL–6 to stimulate the proliferation of primitive human hematopoietic progenitor cells during a period of cocultivation with irradiated cells producing high titers of an ADA-transducing retroviral vector (22). In a series of nine experiments, an average of 82% of the clonogenic progenitors acquired the transferred sequence as determined by analysis using the polymerase chain reaction. In addition, in two experiments, 24% to 44% of the clonogenic progenitors derived from long-term myeloid cultures 9 weeks post-infection were found to contain vector sequence. The transduced ADA enzyme was expressed in both normal and ADA-deficient erythroid colonies and in the nonadherent cells of long-term bone marrow culture for at least 2 weeks at levels that approximate the endogenous ADA levels of normal erythroid cells. More recently, human bone marrow cells maintained in culture on a bone marrow–derived stromal layer were exposed to supernatant containing a marker virus or the ADA virus. In both cases provirus integration was detected in 65% to 70% of the hematopoietic progenitor cells derived from the LTC 5 to 7 weeks later, and in the case of the ADA vector, significant levels of vector-specific transcript were seen (57, 58). The presence of exogenous growth factors did not improve gene transfer efficiency under those conditions, and similar efficiency has been obtained by infecting purified $CD34^+$ cells which are highly enriched in clonogenic cells and HSC (59). These studies demonstrate that stable, high-efficiency gene transfer can be obtained in the most primitive class of human hematopoietic cell detectable in vitro. That efficient transduction of human LTC-initiating cells can be obtained in the absence of virus-producing cells and with

purified cell populations enriched in HSC will greatly facilitate the applicability of this approach to human gene therapy. More direct evidence of efficient transduction of truly primitive human HSC with long-term repopulating ability might come from ongoing gene marking clinical trials. In such trials cancer cells with the potential to contaminate the bone marrow as well as normal bone marrow cells are subjected to genetic marking to track the source of eventual relapses following autologous BMT (1, 2). However, the optimal conditions to mark cancer cells and human HSC may well differ. It is more likely that demonstration of efficient transduction will come from clinical trials aimed specifically at transducing the normal human HSC themselves.

GENE TRANSFER IN HUMAN LYMPHOCYTES Because the development of an efficient gene transfer system for primitive human hematopoietic (stem?) cells and large mammal HSC was laborious and only recently successful (22, 41, 43, 44, 56–59), some investigators took an interest in identifying alternative and more accessible cellular targets for the correction of ADA deficiency. Because T cells are primarily affected by ADA deficiency, they constituted a natural choice in that regard. Potential advantages of using lymphocytes included their ready availability from peripheral blood and their adaptability to tissue culture for expansion, selection, and characterization. ADA-deficient human T and B cell lines had already been shown to produce human ADA at levels similar to normal T and B lymphocytes and to lose their hypersensitivity to 2′-deoxyadenosine following retrovirus-mediated gene transfer of ADA (60). More recent studies evaluated primary lymphocytes as cellular vehicles for gene therapy. Culture-expanded and drug-selected antigen-specific mouse T cells transduced with a NEO^R-ADA vector were shown to persist for several months in the spleens of recipient nude mice, to retain their antigen-specific helper function, and to continue to express the transduced human ADA (61). Also, cultured human tumor-infiltrating lymphocytes (TIL) transduced with a NEO^R vector and selected with G418 retained normal growth characteristics, IL–2 dependence, membrane phenotype and cytotoxic profile (61). Similarly, primary T lymphocytes from an ADA-deficient patient that were successfully transduced with an ADA vector had their sensitivity to 2′-deoxyadenosine restored to normal levels while the nontransduced cells remained hypersensitive (62). The transduced lymphocytes were also found to have the same phenotype, proliferative capacity, and cytotoxic potential as T cells derived from healthy individuals. These studies provided indirect evidence for the safety of transducing lymphocytes from ADA-deficient SCID patients with a defective retroviral vector. Additional

evidence for the relative safety of infusing transduced T lymphocytes in patients came from the first approved human gene transfer experiment (see "Cancer" section below) (63). Finally, lymphocytes from another ADA-deficient patient were shown to survive in immunodeficient mice only if they had first been transduced with an ADA vector (64). Expression of the vector-derived ADA also restored certain immune functions, as was indicated by the presence in reconstituted animals of human immunoglobulin and antigen-specific T cells. This suggested that intracellular expression of ADA in deficient T lymphocytes would confer a survival advantage in vivo even in a detoxifying (ADA normal) environment; this fact provided a strong argument for attempting lymphocyte gene therapy as a supplement to PEG-ADA treatment.

ADA GENE THERAPY FROM LYMPHOCYTES TO BONE MARROW STEM CELLS On the basis of the above studies and a number of other safety studies, a protocol for lymphocyte gene therapy of ADA deficiency (65) and the biological materials to be used in it received approval by the Recombinant Advisory Committee (RAC) and the Director of the National Institute of Health, and by the Food and Drug Administration. According to this protocol, white blood cells are obtained once a month by leukopheresis from ADA-deficient patients who are maintained on PEG-ADA, and mononuclear cells are isolated and grown under conditions that stimulate T cell proliferation (anti-OKT3 antibody and IL–2). Activated T cells are incubated with the vector-containing supernatant and reinfused into the patient after several days of expansion in culture. Two patients have entered this gene therapy trial to date. Both patients have reportedly shown improvement in their clinical condition as well as in a number of in vitro and in vivo immune function studies (e.g. isohemagglutinin titers, skin test for antigen sensitivity, and cytotoxic T cells) (1). In the first patient, the half-life of ADA-corrected T cells appears to have increased severalfold, and the ADA activity of the T cell fraction went from less than 1% of normal to more than 20% during the first 10 months of treatment (eight infusions).

While these results are extremely encouraging, there are some concerns about this strategy. First, since only mature T cells are being transduced, a normal immune repertoire is unlikely to be reconstituted (1, 22, 66). In fact, in both children treated to date, some immune functions have not returned to normal, and positive skin tests are not observed for all antigens (1). Second, even though the risk of insertional mutagenesis is very small with a replication defective retrovirus, the repetitive transduction of millions of cells cumulatively increases that risk. The first concern has led to

modification of the original lymphocyte gene therapy protocol to include, in the future, transduction of both T cells and peripheral blood stem cells (1, 67). Patients will receive granulocyte–colony stimulating factor (G-CSF) for about a week before the collection of white blood cells by leukopheresis, in an attempt to increase the number of circulating stem cells. The $CD34^+$ fraction, enriched in stem cells, and the T lymphocytes will then be transduced with closely related but distinct ADA vectors. Another group in Milan, Italy has received approval from the Italian Committee for Biosafety and has initiated a similar trial involving retrovirus-mediated gene transfer of ADA into peripheral blood T cells and progenitor-enriched bone marrow cells (1, 67). The results of these trials should indicate: (*a*) if true stem cells can be transduced under the proposed conditions; and (*b*) whether transduction of more primitive cells will result in more complete immune reconstitution than transduction of T cells. Of note, both the American and Italian groups are reinfusing the transduced cells without any form of abation of the patient's own marrow. Thus, only a survival advantage of the transduced cells would allow them to expand in vivo. In contrast, on the basis of their successful experiments with ADA-transduction in rhesus monkeys (41), a third group in Rijswijk, Netherlands has received approval from the Dutch National Health Council for bone marrow gene therapy that will include full conditioning of the recipients of transduced progenitor-enriched bone marrow cells.

Although gene therapy of ADA deficiency started by using a different cellular vehicle than the one expected for almost two decades, there are now several indications that clinical trials will be moving toward the use of HSC for the correction of ADA deficiency and other lymphohematopoietic disorders. Refinement in the conditions to obtain, purify, grow, and transduce these cells will probably be defined with time and lead to progressive improvement in results. Nonetheless, gene therapy will almost certainly play a major role in the treatment of ADA deficiency in the future. And, as expected, this rare form of immunodeficiency will have played a central role in the development of an important new form of therapy.

Purine Nucleoside Phosphorylase Deficiency

Purine nucleoside phosphorylase (PNP) deficiency is a rare inherited disease accounting for only 4% of patients with severe combined immunodeficiency (68). While PNP and ADA are both involved in purine metabolism, the phenotypes resulting from their respective deficiency are somewhat different. In PNP deficiency, as in ADA deficiency, T and B cell immunity are affected, and patients usually suffer from recurrent infections beginning in the first year of life. However, T cell function may fluctuate

repeatedly between low and normal, and B cell function is normal in approximately two thirds of PNP-deficient patients. More distinctly, one fourth of PNP-deficient patients come to medical attention for neurological problems, and two thirds have evidence of neurologic disorders, ranging from spasticity to mental retardation. One third of patients develop autoimmune manifestations such as autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura or systemic lupus erythematosus. Like ADA, PNP is found in most tissues of the body but is at highest levels in lymphoid tissues, which explains why the lymphoid system is predominantly affected. Similar to the effect of dATP in ADA deficiency, the elevated level of dGTP found in PNP deficiency is thought to inhibit ribonucleotide reductase and thus to impede cell division. Depressed GTP levels may correlate with neurologic dysfunction. The prognosis of PNP deficiency is also very poor as no patient has reached the third decade of life. Many therapies have been attempted with inconsistent results, including BMT which is currently considered the treatment of choice.

There has been only one report to date concerning the development of retrovirus-mediated gene transfer of PNP (69). A human PNP cDNA was placed under the control of a mouse metallothionein promoter in a retroviral vector. While substantial human PNP activity was detected in virus-infected mouse fibroblasts by isozyme analysis, integrated provirus failed to express the human isozyme in spleen colony tissue following BMT with transduced marrow cells. This poor result reflected the inability of that specific construction to direct efficient in vivo expression in hematopoietic cells. Although this is certainly a correctable problem, the fact that PNP is so rare [33 patients have been reported; at least 29 of these are dead (68)] probably explains the relative lack of effort in developing gene therapy for this disorder. Another aspect which may limit the applicability of gene therapy to PNP deficiency is the existence in many patients of neurological manifestations which may not respond to gene supplementation in the hematopoietic system. However, even neurological deficits might improve if bone marrow gene therapy was performed at an early age, before permanent neurological damage occurred from metabolic imbalance. The potential responsiveness of neurological deficits to bone marrow gene therapy, at any rate, could be evaluated by performing early allogeneic BMT in patients with a suitable donor and, eventually, through the creation, by gene disruption, of a mouse model of PNP deficiency in which to test the gene therapy approach (70). In conclusion, while gene therapy would in theory be applicable to at least those cases of PNP deficiency that do not have serious neurological manifestations, the rarity of the disorder might be the principal deterrent to the development of this labor-intensive approach.

Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency (LAD) is a rare autosomal recessive disorder caused by derangements in CD18 expression and characterized by profound abnormalities in leukocyte function resulting in recurrent, life-threatening infections (71). The abnormality in CD18 expression results in the deficiency of three types of leukocyte adhesion molecules that are heterodimers normally formed between CD18 and one of three CD11 sub-units: LFA–1 (CD11a and CD18), Mac–1 (CD11b and CD18), and p150,95 (CD11c and CD18). The potential for significant clinical improvement with partial reconstitution of the gene function and the availability of an authentic canine model of the disease should facilitate the development of gene therapy for LAD. A CD18 transducing vector was first used to transfer the normal coding sequence of CD18 into lymphocytes from patients with LAD (72). While only 2–4% of the cells expressed detectable levels of LFA–1 on the cell surface following retroviral transduction, these cells could be enriched by sorting for cell surface expression of CD11a and CD18. These purified cells expressed normal amounts of LFA–1 on their surface and demonstrated reconstitution of LFA–1–dependent adhesive function. Further to this initial study, retrovirus-mediated gene transfer of CD18 was evaluated in vivo in mice following transplantation of transduced bone marrow cells (73). Of 60 surviving mice 25 showed significant expression of human CD18 on their leukocytes. Expression of human CD18 was consistently highest and most frequent in granulocytes and reached levels that should be therapeutic in LAD-derived granulocytes. This is fortunate since granulocyte dysfunction appears to be the most important cellular abnormality in LAD (71). Also, vector-derived CD18 demonstrated appropriate post-transcriptional regulation in transduced murine granulocytes in response to a relevant physiological stimulus, activation of protein kinase C. Finally, transduction of murine HSC with a vector directing constitutive expression of human CD18 had no apparent effect on hematopoietic reconstitution and was not associated with obvious disease.

It therefore appears that the prerequisites to proceed with a clinical trial of gene therapy for LAD will soon be met. Recent success in retroviral transduction of long-term repopulating marrow cells of dogs (43) indicates that it might be possible to test the gene therapy approach in a canine model of the disease. However, presently attainable levels of expression of nonselectable transduced genes in dogs might be insufficient to evaluate the therapeutic benefit of this strategy. In that case, it would be appropriate to proceed with a clinical trial of gene therapy nonetheless, provided that current bone marrow gene therapy and gene marking experiments demonstrate efficient and safe gene transfer into human HSC.

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is characterized by recurrent life-threatening bacterial and fungal infections and excessive granuloma formation. Neutrophils, eosinophils, monocytes, and macrophages of CGD patients have deficient microbicidal activity because they fail to undergo a respiratory burst when stimulated (74, 75). The products of the respiratory burst, which include superoxide and hypochlorous acid, play a critical role in killing pathogenic bacteria, fungi, and parasites. While all CGD patients share the same functional defect, there is heterogeneity in the molecular basis of the disorder. The enzyme that catalyzes the respiratory burst, NADPH oxidase, consists of at least four subunits: $gp91^{phox}$ and $p22^{phox}$, the two subunits of membrane cytochrome b_{558} which is the terminal electron carrier of the oxidase, and $p47^{phox}$ and $p67^{phox}$, two cytosolic oxidase components. The X-linked form of the disorder, which accounts for approximately 60% of the cases, involves a defect in the gene encoding the large subunit of cytochrome b_{558}, the $gp91^{phox}$. The most common autosomal form of CGD, responsible for 30% of all cases, results from a failure to produce the 47-kDa cytoplasmic oxidase component, $p47^{phox}$. Deficits in the two other components are inherited as autosomal disorders and account for the reminder of the cases. BMT has been curative in CGD (76), indicating that the transfer of genetically corrected autologous HSC should correct the disorder. Recent clinical trials have demonstrated that γ-interferon can ameliorate CGD (74, 75, 77), but somatic gene therapy would offer the potential for a complete and permanent correction of the disease without the risks involved in allogeneic BMT. Because the cDNA encoding each subunit of the oxidase have been cloned, the feasibility of somatic gene therapy can be evaluated experimentally, although the lack of an animal model and of cell lines derived from phagocytic cells of CGD patients complicates the evaluation of efficacy.

There has been no report yet on a gene therapy experiment using the cDNA encoding the $gp91^{phox}$ deficient in the most common, X-linked form of CGD. A recent report, however, described the use of a retroviral vector to express the $p47^{phox}$, deficient in the most common autosomal form of the disease (78). A $p47^{phox}$-transducing retroviral vector was used to infect promyelocytic (HL60) and monocytic (U937) human leukemia cell lines as well as an Epstein Barr virus–transformed B lymphocyte cell line (EBV-BCL) derived from a $p47^{phox}$–deficient CGD patient. EBV-BCL is the only type of immortal cell line available from CGD patients that expresses the components of the NADPH oxidase system. Unfortunately, compared to granulocytes or macrophages, the activity of the oxidase system is miniscule even in normal EBV-BCL, making it difficult to detect restoration of

the overall oxidase activity in these cells. Nonetheless, transduced $p47^{phox}$ messenger RNA (mRNA) and protein were detected in all transduced cell lines. The amount of recombinant $p47^{phox}$ protein produced within these cells was greater than the native $p47^{phox}$ in uninduced HL60 or U937, but substantially less than is present in normal neutrophils, induced HL60 cells, or even normal EBV-BCL. Some evidence suggests that this low level of expression was due to the specific retroviral vector used in these experiments. The predominant retrovirus transcripts containing $p47^{phox}$ in most cells were less than the predicted full-length, while protein production seemed to correlate with the amount of full-length transcript. This may have resulted from alternate splicing or from the production of truncated versions of the mRNA from the proviral sequence. It is likely that alternate vector constructions will result in improved transduced $p47^{phox}$ expression in hematopoietic cells. Once potentially therapeutic levels of transduced expression are obtained in vitro in cell lines and in vivo in mice or other animals, it would be justifiable to obtain bone marrow from CGD patients for the preclinical evaluation of somatic gene therapy. In theory, efficient transduction of the $gp91^{phox}$, deficient in the X-linked form of the disease, should also be possible. Thus, the most common forms of CGD should be amenable to somatic gene therapy in the foreseeable future.

X-Linked Immunodeficiencies

Apart from the X-linked form of CGD, X-linked immunodeficiencies include X-linked agammaglobulinemia (XLA), X-linked SCID (XSCID), Wiskott-Aldrich syndrome (WAS), X-linked lymphoproliferative disorders syndrome, and X-linked hyper IgM syndrome. It has been suggested that some or all of the genes involved in these disorders might be members of a gene family (79). While obligate carriers of these disorders are normal by all immunologic criteria, this has been ascribed to the preferential use of the normal, nonmutant X as the active X chromosome in the cell lineages affected by the gene defect in XLA, XSCID, and WAS. X-linked immunodeficiencies are potential candidates for somatic gene therapy once the genes involved are cloned, but it is not certain whether each of these defects would behave as a true recessive disorder, because the mutation on an actively transcribed X chromosome could in theory have a dominant effect. In the case of XLA, the correction of the molecular defect in B lymphocytes by cell fusion does indicate that this is a true recessive disorder (80). Evidently this and other aspects of the feasibility of somatic gene therapy for X-linked immunodeficiencies will be addressed properly when the genes involved are isolated.

ACQUIRED DISORDERS OF THE IMMUNE SYSTEM

Acquired Immunodeficiency Syndrome

Although somatic gene therapy was initially conceived as an approach to treat severe inherited disorders, it is now apparent that it will eventually find its most common applications in acquired disorders such as cancer and infectious diseases. Given the difficulty in developing standard immunization and efficient therapeutic agents against the human immunodeficiency virus (HIV), the retrovirus that causes the acquired immunodeficiency syndrome (AIDS), the suggestion was made that HSC from an infected individual could be made resistant to HIV infection by gene transfer (81). The genetically modified cells would presumably have a proliferative advantage once transplanted into the donor, and thus they could reconstitute an HIV-resistant hematopoietic system. Alternatively, a co-expressed, selectable gene could be included in the vector if selection by HIV-resistance was not sufficient. Since the hematopoietic system is the principal target and reservoir of HIV in the organism, this form of "intracellular immunization" would have a real chance of success (81). Several approaches for inhibition of HIV replication have been proposed and investigated experimentally. Mutant HIV proteins exercising a dominant negative effect on HIV replication (82, 83), antisense RNA (84, 85), and RNA that competes for the HIV promoter or trans-activation responsive (TAR) element (86) all required constitutive high-level expression to block HIV replication. This is probably why they did not result in complete inhibition of viral replication. While these strategies may still be applicable to the treatment of HIV, alternative approaches that have been investigated recently are reviewed in more detail here.

EXPRESSION OF SOLUBLE CD4 The helper/inducer T cell antigen CD4 is the cell surface receptor mediating entry of HIV into the cell (87), and several groups have shown that soluble forms of the CD4 antigen can bind to and inhibit HIV infection of $CD4^+$ cells. Efficacious treatment of AIDS may, however, entail continuously maintained levels of soluble CD4. Toward this end, one group has investigated the use of retrovirus-mediated gene transfer to produce a truncated, soluble form of CD4 (88). Direct physical interaction was demonstrated between the truncated CD4 molecule produced by transduced cells and the partially purified HIV envelope glycoprotein (gp120). Also, cocultivation of cells producing the soluble CD4 and HIV-susceptible cells resulted in a four- to ten-fold decrease in infectivity when the mixture was challenged with two independent isolates of HIV–1. It was suggested that soluble CD4 could be produced in vivo in AIDS patients either by retroviral transduction of the hematopoietic system or by implantation of transduced fibroblasts or endothelial cells.

Another group explored a slightly different approach (89). They showed that expression of a mutated soluble CD4 molecule containing a specific retention signal for the endoplasmic reticulum blocked the secretion of HIV gp120 and surface expression of gp120/41. By blocking the transport of the HIV glycoprotein, the retained CD4 molecule prevented the cytopathic fusion of CD4 cells that is normally caused by the HIV glycoprotein. The authors also speculated that retention of the complex of HIV glycoprotein precursor (gp160) and mutated CD4 in the endoplasmic reticulum should inhibit assembly of infectious virus at the plasma membrane. Another potential advantage of this strategy is that the HIV should be unable to evade the block in glycoprotein transport without losing the ability to bind its receptor. While this constitutes an interesting approach to inhibiting HIV, the above experiments were conducted with transfected DNA vectors in which the mutated CD4 DNA was placed under the control of the bacteriophage T7 promoter. Efficient expression of the mutated CD4 was then obtained by infecting the transfected cells with a recombinant vaccinia virus that expressed the T7 RNA polymerase. Because this system is not directly transposable to somatic gene therapy, it will be necessary to reproduce these results and show inhibition of HIV replication with a vector that can be used for somatic gene transfer before this strategy can be contemplated in AIDS patients.

HIV-INDUCIBLE CELL DEATH Inhibition of HIV infection and propagation could also be achieved by introducing HIV-inducible genes that would directly or indirectly cause cell death upon infection by HIV. The advantage of this approach is that it would not require sustained expression of a product inhibitory to viral replication, with potential detrimental effects on the cell, but would produce transient and possibly low level expression of the inhibitory gene. This strategy takes advantage of the existence of the transactivator of transcription (tat) protein which, by acting both transcriptionally and post-transcriptionally, induces HIV–1 gene expression up to 1000-fold. The tat-responsive RNA sequence, the TAR element, is found at the 5′ end of all HIV–1 messenger RNAs. Tat appears to act by preventing premature termination of transcription of nascent HIV transcripts and by increasing the rate of initiation and elongation of transcription. Heterologous genes will be inducible by tat if the TAR element is included at the required position in the construction. Genes whose products are directly cytocidal (suicide genes) and genes that can indirectly induce cell death have been evaluated for use in anti-HIV gene therapy.

Suicide genes Several groups are investigating "suicide" strategies to combat HIV-mediated pathogenesis. Induction of a suicide gene when a

cell is infected by HIV would eliminate the initial pool of infected cells and thus prevent disease progession. One group used a replication-defective adenovirus vector expressing the herpes simplex virus type 1 thymidine kinase (*tk*) gene under the control of the HIV–1 long-terminal repeat (90). Infection of tat-expressing human cell lines with the vector resulted in high-level *tk* expression, which was not deleterious to the viability of these cells. In the presence of the antiherpetic drug ganciclovir, which becomes toxic after phosphorylation by *tk*, infection with the *tk* vector resulted in a massive reduction in the viability of tat-expressing cell lines. Because adenovirus vectors have not been shown to result in stable gene transfer, retrovirus vectors might be more suitable for this form of therapy. Another group stably transfected the *tk* gene under the control of the HIV–1 promoter and TAR element in $CD4^+$ lymphoid cells (91). Upon HIV infection, high level of expression of *tk* resulted in death of HIV-infected cells and arrest of HIV spreading when cells were cultured in presence of acyclovir, another antiherpetic drug. Complete protection of the *tk*-expressing cells was obtained using acyclovir concentrations that are commonly detected in the plasma of patients treated for herpes simplex virus infection.

Another gene that has been studied in suicide strategies is the diphtheria toxin A chain (DT-A) gene. DT-A is an extremely potent inhibitor of protein synthesis in eukaryotic cells. While the activation of antiherpetic nucleoside analogs by *tk* is dose-dependent and compatible with cell viability at low basal level of expression, the toxicity of DT-A requires tightly regulated expression. It has been estimated that 1 molecule of DT-A per cell is sufficient to kill murine L cells. Because significant basal expression was observed following transfection of a plasmid construction that expressed DT-A under control of the HIV–1 long-terminal repeat, *cis*-acting negative regulatory elements from the *env* region of HIV–1 genome were inserted in the 3′ untranslated region of the construct (92). This markedly reduced basal expression of DT-A and made the presence of both HIV regulatory proteins, tat and the regulator of expression of virion proteins, rev, necessary for maximal expression. Clonal isolates of a human cell line transfected with this tat- and rev-inducible DT-A construction were recently shown to generate 50% to 96% less secreted viral p24 antigen (the *gag* gene product) than control cells, following transfection with an HIV provirus (93). Although this level of inhibition may not be therapeutically sufficient, the authors argued that the large number of viral genomes entering a cell by DNA transfection may lead to an underestimation of the efficiency with which the production of viral structural proteins would be inhibited in a genuine infection. The success in generating cell lines with the DT-A construction also indicated that extremely low basal expression had been achieved.

Induction of cytocidal immune response An alternative approach to intracellular immunization would be to introduce HIV-inducible genes encoding products capable of eliciting a cytocidal immune response. Genes encoding products to which a person typically has developed immunity via natural exposure or vaccination are candidate for that use. For instance, a fragment of the influenza A virus hemagglutinin (H5 HA) gene is capable of eliciting antibody and cytotoxic T cell responses. A tat-dependent HIV-based retroviral vector conferring H5 HA expression was shown to express detectable amounts of H5 HA in human epithelial cells only in presence of tat expression (94). However, because available anti-H5 HA cytotoxic T cells are murine major histocompatibility–restricted, the effect of the H5 HA vector on both HIV infection and the response of cytotolytic cells could not be evaluated.

Ribozymes are small RNA molecules that allow sequence-specific endoribonucleolytic cleavage in a catalytic manner (95). Ribozymes of the so-called "hammerhead" catalytic motif have been the most studied for anti-HIV applications, but ribozymes of the alternate "hairpin" motif could also be used. The ribozyme of satellite tobacco ringspot virus, on which several anti-HIV ribozymes have been based, contains a catalytic domain of 24 conserved nucleotides that is flanked on both sides by sequences at least 8 nucleotides long, complementary to the region surrounding the cleavage site. Cleavage of target RNA occurs after a GUC, GUU, or GUA sequence, and involves hydrolysis of a 3′–5′phosphodiester bond. Because the catalytic RNA is not consumed during the cleavage reaction, a large number of substrate molecules can be processed. Although ribozymes can be exogenously delivered to cells, the most effective means of delivery in vivo would almost certainly be by gene transfer.

In one study, $CD4^+$ human epithelial cells were stably transfected with a vector expressing a hammerhead ribozyme targeted to HIV–1 *gag* transcripts under the control of a human β-actin promoter (96). Following challenge with HIV–1, a significant reduction in the level of HIV–1 *gag* RNA, and a 20- to 40-fold reduction in p24 production was observed in comparison to cells not expressing the ribozyme. No deleterious effect from the expression of the anti-HIV ribozyme could be detected after several months in cell culture. In an approach more directly applicable to gene therapy, another group constructed retroviral vectors expressing an HIV–1 5′ leader sequence-specific ribozyme and used them to infect a human $CD4^+$ lymphocyte cell line (97). Vectors allowing constitutive expression of the ribozyme from various viral promoters resulted in variable degrees of resistance to HIV infection. In contrast, no HIV–1 (p24) production was detected for the entire duration of the experiment following challenge of cells transduced with a vector containing a fusion *tk*-TAR

promoter and allowing ribozyme expression in both a constitutive and tat-inducible manner.

As with most treatments for HIV infection, one potential limitation to the use of ribozymes is the high mutability of HIV–1 which could render viral RNA resistant to ribozymes. However, this problem should be preventable if highly conserved sequences are targetted such as those coding for the CD4 interaction site of *env*, or functionally important segments of *pol*, *tat*, and *rev*, or important response elements such as the binding sites for *tat* and *rev* (98).

PRACTICAL CONSIDERATIONS A number of factors must be considered before an anti-HIV gene-therapy approach can be attempted in patients. These include: (*a*) the potential toxicity to normal cells; (*b*) the ability to deliver and express sufficient levels of the inhibitory gene in the target cell population; (*c*) the effect of the inhibitory gene on in vivo replication and propagation of the virus; and, above all, (*d*) the impact of this approach on the preservation of immune functions in HIV-infected individuals. The aspect of toxicity of anti-HIV gene therapy is particularly relevant to the suicide strategy. Depending on the toxicity of the suicide gene, the significance of basal, tat-independent expression, and of alternative mechanisms for activation of HIV enhancer in T cells (99) will have to be carefully assessed. It is likely that the addition of *cis*-acting regulatory elements to the vector, such as the rev-responsive element, will result in adequate control of expression (90, 92). In regard to the delivery and expression of anti-HIV genes, several groups have developed defective HIV–1 retroviral vectors and have suggested that these would be ideal vehicles for gene transfer into $CD4^+$ cells (100–102). Because $CD4^+$ lymphocytes are short-lived, transduction of these cells would only have a temporary effect and would probably need to be repeated at regular intervals. As originally suggested, intracellular immunization might be best achieved by stable transduction of HSC allowing reconstitution of an HIV-resistant hematopoietic system (81). This now appears to be feasible.

The impact of a given anti-HIV gene therapy on viral propagation and on the preservation of immune functions would best be evaluated in vivo in animal models. In the case of the suicide strategy, for instance, the overall efficacy would ultimately depend upon how quickly the infected cell could be destroyed or virus production could be inhibited relative to the time required for progeny virions to be formed and to propagate (94). It is conceivable that enhanced destruction of the cellular target of HIV mediated by the suicide gene would lead to even more rapid loss of the patient's immune functions if cell death was not accompanied by complete inhibition of virus propagation. Given the possibility of infecting non-

human primates with HIV or simian immunodeficiency virus (103), and of transducing primate HSC with retroviral vectors (41), one could test anti-HIV gene therapy strategies in these species. Alternatively, immunodeficient mice reconstituted with transduced human hematopoietic cells could be used (104, 105). The decision to proceed to human trials with or without the benefit of in vivo animal data will ultimately have to be based on a careful assessment of potential risks and benefits of the procedure in light of the clinical condition of the patients.

CLINICAL TRIAL One of the most recently approved human gene transfer experiments is a gene marking protocol in AIDS patients undergoing allogeneic bone marrow transplantation for non-Hodgkin lymphoma (106). HIV seropositive patients with lymphoma will receive bone marrow from an HLA-identical related donor after high-dose chemotherapy and total body irradiation to eradicate the lymphoma and the HIV infected lympho-hematopoietic system of the patient. AZT and patient-derived $CD8^+$ HIV *gag*-specific T cells expanded in culture will be administered post transplantation in an attempt to prevent infection of the incoming marrow by residual HIV in the patient. Before they are infused, these HIV-specific T cells will be transduced with a retroviral vector expressing a bifunctional protein encoded by an hygromycin phosphotransferase-*tk* fusion gene (107). Vector-transduced cells can thus be positively selected for hygromycin resistance before their administration, and negatively selected in vivo (if necessary) by antiherpetic nucleoside analogs such as ganciclovir. The justification for gene marking/suicide in this experiment is two-fold (106). First, because the HIV specific $CD8^+$ cells could potentially attack not only $CD4^+$ lymphocytes, but also other HIV-infected cells such as CNS microglial cells, alveolar macrophages and, possibly, hematopoietic progenitors, unique toxicities not usually observed with adoptive T cell transfer could be observed with the administration of increasing doses of these cells. The suicide *tk* gene would in theory permit eradication of HIV-specific T cells if there were unacceptable toxicities or other deleterious effects. Second, it is possible that these host-derived T cells will be rapidly eliminated by donor lymphocytes due to recognition of minor histocompatibility antigens. Retroviral gene marking will provide a sensitive indicator of the in vivo survival of infused T cells. Although this does not constitute a gene therapy experiment per se, i.e. the transferred gene will not contribute directly to the treatment of HIV infection, it constitutes an important first step in applying gene transfer to the treatment of AIDS. As in the case of cancer treatment (see below), gene marking experiments will undoubtedly, and probably shortly, be followed by gene therapy protocols.

Cancer

Another common acquired disorder that may be amenable to some form of somatic gene therapy is cancer. In fact, although cancer has relatively recently become an experimental target for somatic gene therapy, five out of nine gene marking protocols and five out of eight gene therapy protocols that have been approved by the RAC involve gene transfer in cancer patients. Because the pathophysiology of most cancers appears to involve, at some stage, the activation of dominant oncogenes and/or the loss of function of tumor suppressor genes, the most obvious approach to gene therapy of cancer would be to inhibit the function of the activated oncogene or to introduce a normal copy of a tumor suppressor gene. There are in fact several examples of suppression of the malignant phenotype in vitro, and in certain cases in vivo in nude mice, by transfer of tumor suppressor genes or of other genes (such as cell adhesion molecules) that affect the differentiation or the metastatic potential of malignant cells (see 108 for review). Also, antisense oligonucleotides and ribozymes targeted to oncogenes have been shown to alter the proliferative potential of malignant cells in vitro, and their tumorigenicity or metastatic potential in animals. The main limitation of this approach is our current inability to target very efficiently and specifically malignant cells in vivo for gene transfer. Considering that cancer will often relapse after several cycles of chemotherapy each cycle of which achieves a tumor cell kill of 99.9% or more, the efficiency of in vivo gene delivery would need to be far beyond what we can currently achieve to apply this form of gene therapy. Other forms of gene therapy, however, would not require such high efficiency of gene transfer. These include genetic immunomodulation, specific activation of prodrugs in tumors, and normal tissue protection (108).

GENETIC IMMUNOMODULATION The transfer to the tumor-bearing host of immunological reagents, such as immune cells, that have antitumor reactivity and can mediate direct or indirect antitumor effects has been described as adoptive immunotherapy. Adoptive immunotherapy of cancer has been attempted with lymphokine-activated killer (LAK) cells and with tumor-infiltrating lymphocytes (TIL) (see 109 for review). TIL are isolated by culturing single cell suspensions from tumors in IL–2. In the mouse, TIL are exclusively $CD8^+$ and show killing activity for tumor cells which is both MHC class I–restricted and dependent on the recognition of specific tumor antigens. Titration of TIL in mouse tumor models indicated that TIL were 50 to 100 times more potent than LAK cells in reducing tumor metastases. There is also evidence that the human TIL, which include both $CD4^+$ and $CD8^+$ cells, recognize specific antigens that can be shared between tumors. Immunotherapy with TIL and IL–2 was

shown to produce measurable responses in up to 30% of melanoma patients.

Recently, gene transfer has been used both to study and to improve adoptive immunotherapy. In fact, the first approved human gene transfer experiment involved retroviral gene marking of tumor-infiltrating lymphocytes (TIL) in patients with advanced melanoma (63). In the gene transfer experiment, a Neo^R gene was delivered to autologous TIL by retroviral infection, genetically marking these cells and allowing study of their long-term distribution and survival. The results of that experiment indicated that: small numbers of transduced cells persisted in the circulation of all patients for at least 3 weeks, and up to 2 months in some cases; transduced cells were recovered from tumor deposits in 3 of 5 patients, from 6 days to 2 months after their administration; and, most importantly, no ill effect of the gene transduction was noted, and all safety studies were negative, including tests for the presence of live virus production. Although these observations were made on a small number of patients and with a short follow-up, they provided the first direct evidence of the safety and efficiency of retrovirus-mediated gene transfer in humans (63, 66).

Cytokine gene transfer in lymphocytes Following this relatively successful experiment of gene transfer into TIL, the proposal was made to attempt increasing the antitumor activity of TIL in advanced melanoma by transducing them with a vector for tumor necrosis factor (TNF). TNF is a cytokine that possesses a wide variety of biologic activities including potent anti-tumor activity and immunomodulatory properties (110). However, the maximum tolerated dose of TNF in humans is only 2% of the dose required to mediate antitumor effects in the mouse. The rationale of the TIL/TNF protocol was to transduce TIL with a TNF vector, hoping that TIL cells would deliver high local concentration of TNF in tumor deposits and induce immune-mediated destruction of the tumor cells. However, it has proven difficult to achieve consistently high levels of cytokine production in lymphocytes following retrovirus-mediated gene transfer, possibly because of post-transcriptional control of cytokine expression in this cell type. Even in selected patients, the level of expression obtained in TIL was still only in the range of low TNF-producing tumor clones which only rarely (5%) induced tumor regression in mice (109, 110). The TIL/TNF protocol was approved for 50 patients and initiated in January 1991. As of June 1992, no side effect has been observed in patients infused with as many as 10^{11} TNF-modified TIL (1, 109). Insufficient numbers of patients have been treated to date to determine if TNF/TIL and IL–2 is superior to TIL and IL–2 in the treatment of advanced melanoma. Additional genes

being studied for insertion into TIL to improve their antitumor activity include γ-interferon, IL–2, IL–6 and genes for chimeric T cell receptors (109).

Gene transfer into tumor cells Another approach to genetic immunomodulation is the introduction of genes into tumor cells that will induce an immune reaction against modified and unmodified tumor cells. Experimental evidence suggests that the expression of exogenous cytokines by tumors will, in certain cases, reduce the tumorigenicity of cancer cells. Tumor cells transduced or transfected with a vector expressing γ-interferon (IFN) (111–113), tumor necrosis factor-α (TNF) (110), IL–2 (114, 115), IL–4 (116, 117), or GM-CSF (118) will be less tumorigenic in mice because of increased anti-tumor host immunity. In several of these experimental models, injection of a mixture of cytokine-secreting and parental tumor cells results in killing of *both* populations, and injection of cytokine-secreting cells generates long lasting immunity against the parental tumor cells. However, results in animals were variable, and the applicability of these findings to human treatment remains to be established.

Two protocols have been initiated in which tumor cells from patients with advanced metastatic cancer are transduced with either a TNF or IL–2 vector and used for immunization to collect potentially more active TIL (119, 120). In these protocols, the cytokine-transduced autologous tumor cells are injected subcutaneously and intradermally into the patient's upper thigh. Three weeks later, the injection site and draining lymph nodes are removed, lymph node T cells are expanded in culture and returned to the patient with IL–2. No data are yet available on these studies.

Because transfection of MHC class I antigens was shown to increase the anti-tumor activity of murine TIL against both the modified and unmodified tumor cells, another potential approach to immunomodulation is to introduce MHC class I expression vectors into tumor cells. One group is exploring this approach by injecting a plasmid DNA vector for HLA-B7 complexed with liposome directly into tumors of melanoma patients (121). If this method of gene transfer proves efficient, it would provide an attractive alternative to the more complex ex vivo modification of tumor cells.

PRODRUG ACTIVATION Genes that result in the conversion of nontoxic prodrugs to toxic active forms could be directed to cancer cells in two ways: the "suicide" gene could be placed under control of a promoter/enhancer that would be active in tumor cells specifically; or the suicide gene could be specifically delivered to the tumor cells. The activation of a prodrug by a vector that is specifically expressed in tumor cells has been called "virally directed enzyme prodrug therapy" (VDEPT).

Vectors expressing a herpes simplex *tk* gene under the control of an α-fetoprotein or albumin promoter were shown to be specifically expressed in hepatoma cells and normal hepatocytes, respectively. Because the thymidine kinase can convert the nontoxic prodrug 6-methoxypurine arabinonucleoside (Ara-M) to the phosphates Ara -AMP, -ADP, and -ATP which are potent cytotoxic agents, *tk*-vectors under control of the α-fetoprotein promoter could be used to sensitize hepatoma cells to the prodrug. This model is currently being tested in mice. Genes that show increased expression in tumors of other organs could potentially provide promoters that could be used in similar strategies (108).

Although in vivo targeting for gene delivery can be accomplished with certain cell types, such as hepatocytes (122), by using cell surface receptors specifically expressed by these cells, this is not yet a widely applicable strategy. Alternative methods for delivery of suicide genes into tumor cells include intratumoral injection of cells producing a retroviral vector, or injection into the tumor site of lethally irradiated tumor cells that have been transduced with a suicide vector. The first approach has given encouraging results in the treatment of brain tumors in animals (123). The second approach, in which the genetically modified cells would presumably affect neighboring tumor cells by activating a toxin and possibly inducing an immune reaction, has now been approved for a clinical trial in patients with ovarian cancer (1, 2).

NORMAL TISSUE PROTECTION Stomatic gene transfer could be used in the treatment of cancer to confer protection against the toxicities of anticancer drugs. Because hematotoxicities are often dose-limiting in cancer treatment, the hematopoietic system would be the prime target in this approach. Protection of the hematopoietic system against chemotherapeutic agents would also permit the intensification of anti-cancer treatment with potentially improved responses and survival. Protection of the hematopoietic system could be obtained by transferring drug resistance genes. Both a mutant form of dihydrofolate reductase and the human multidrug resistance–1 gene have been shown to increase the tolerance of mice to the corresponding drugs following introduction of the drug-resistance genes in the hematopoietic system (124, 125). A less specific protection could also be obtained by transferring genes for hematopoietic growth factors. Transduction of hematopoietic cells with a granulocyte/macrophage-colony-stimulating factor vector increases the tolerance of mice to cytotoxic drugs (126). However, because transduction of hematopoietic cells with hematopoietic growth factors could in theory lead to autonomous growth, transduction of other cell types such as fibroblasts or peripheral blood lymphocytes (127) would probably be more desirable.

In conclusion, several avenues are being investigated to apply somatic gene transfer to cancer treatment. Although none of these approaches are likely to result in cures if used alone, it is plausible that some of them may eventually play a role, along with more traditional forms of therapy, in the treatment of certain types of cancer. Simplification of the techniques of gene transfer would likely be required, however, to make gene therapy widely applicable to a common disorder such as cancer.

CONCLUSION

Human somatic gene therapy has become a reality. As expected, the first disorder to be treated by gene therapy was a severe form of immunodeficiency, adenosine deaminase deficiency. Gene therapy will almost certainly play an important role in the treatment of other primary immunodeficiencies, notably leukocyte adhesion deficiency and chronic granulomatous disease. Important progress has also been made toward achieving efficient and stable gene transfer to liver, lung, or muscle cells. This will result in the application of somatic gene therapy to inherited disorders such as the muscular dystrophies, cystic fibrosis or α_1-antitrypsin deficiency. However, gene therapy may eventually be more commonly applied to acquired disorders such as AIDS and cancer. In both cases, promising strategies are being evaluated experimentally, and clinical trials have been initiated. While it remains difficult to predict the role that gene therapy will play in these or other disorders, continuous progress will surely make it applicable to an increasing number of diseases.

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T CELL–DEPENDENT B CELL ACTIVATION

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Abstract

B cells obtain help from T cells in the antibody response by acting as antigen-specific antigen presenting cells. A direct signal through binding of antigen to membrane Ig can enhance B cell antigen presentation and T-dependent B cell activation, but is not required for a productive interaction between a small resting B cell and a differentiated helper T cell. As a result of helper T cell recognition of antigen on the B cell surface, the T cell becomes activated and in turn activates the B cell. T cell help has two components: lymphokines which act as growth and differentiation factors for B cells, and additional signals which require cell contact and enable B cells to respond to lymphokines. Contact help activity is regulated like lymphokine synthesis and secretion. Because contact help activity is retained by fixed, activated helper T cells and plasma membranes prepared from activated T cells, contact help is likely to be owing to new proteins expressed as membrane-bound lymphokines or activation antigens on helper T cells. Once induced, contact help can be delivered to B cells independently of recognition of antigen/class II MHC. A newly identified activation antigen of helper T cells, a ligand for the B cell differentiation antigen, CD40, is a key component of contact help. The roles of other T and B cell membrane molecules in contact help are reviewed.

INTRODUCTION

That the antibody response to protein antigens requires specific recognition of the same antigen particle by both B lymphocytes and T lymphocytes

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has been known for many years. B cells are the precursors of the antibody secreting cells, and T cells provide help. The mechanism of T cell help for B cells has been actively investigated, and much progress has been made (1, review), but a satisfactory description of T cell help remains elusive.

The pioneers of in vitro antibody responses discovered that mitogen or antigen-activated T cells secrete soluble molecules which can replace T cells in supporting antibody responses to red cells. It was proposed that local action of such factors could explain antigen-specific collaboration between T cells and B cells. This line of investigation led eventually to the identification of the lymphokines that act as growth and differentiation factors for B cells.

Other in vitro studies showed clearly that the requirement for T cells in the response to limiting concentrations of antigens cannot be replaced by lymphokines but requires antigen-specific, linked recognition of the same antigen particle by T cells and B cells, as does the antibody response in vivo. Also as previously shown in vivo, this interaction is restricted by allelic polymorphism at the major histocompatibility complex (MHC) (2, 3, 4—review). In spite of much effort, and a long detour through antigen-specific, MHC-restricted helper factors, the beginnings of an accurate scheme of T/B collaboration in the antibody response had to wait for a better understanding of how T cells recognize antigen and require appropriate MHC alleles on the cells with which they interact. We know now that T cells recognize antigen only on cell surfaces as peptide fragments bound to MHC molecules. B cells get help from T cells by acting as antigen-specific antigen presenting cells (APCs). Antigen recognition is sequential rather than simultaneous: First the B cell binds antigen with its antigen receptor membrane immunoglobulin (Ig) and internalizes and degrades the antigen; then it presents peptides from the antigen on the cell surface bound to class II MHC molecules; finally the T cell recognizes the processed antigen on the B cell surface, and mutual activation results. This scheme explains MHC restriction of T cell/B cell collaboration, as well as the ability of T cells recognizing internal peptide determinants to help B cells make antibodies that react with proteins only in their native conformation. This review begins with an introduction to B cells as APCs and then takes up the question: How does the T cell activate the B cell when it recognizes antigen on the B cell surface? Much of the evidence in this review is drawn from T cell–dependent polyclonal B cell responses in vitro.

B CELLS AS ANTIGEN PRESENTING CELLS

As soon as the outlines of how MHC molecules guide T cell recognition of antigen on cell surfaces became apparent, it was proposed that B cells

get help by acting as APCs (5, 6). Unfortunately, B cells failed to show APC function in vitro with primed T cells or T cell lines. In part, this was a technical problem, since APC populations were routinely irradiated to prevent them from contributing to the proliferative response of the T cells, and the APC function of resting B cells is radiation-sensitive (7). In fact, when the B cells were not irradiated, they responded polyclonally following polyclonal antigen presentation to antigen-specific helper T cell lines (8, 9). But resting B cells are also defective in the costimulatory signals that T cells require in order to proliferate following antigen recognition (10, review). Therefore, the first reports of B cells as stimulatory APC involved B cell tumor lines and normal B cells stimulated with anti-Ig (11).

Antigen Receptor–Mediated Antigen Presentation

The unique feature of B cells as APCs is their ability to use their clonally distributed antigen receptors to take up antigen into the processing pathway. Antigen processing and presentation mediated by membrane Ig was first demonstrated using normal B cells to present rabbit anti-mouse Ig to rabbit Ig-primed T cells, resulting in a T cell–proliferative response (11). Antigen-specific antigen presentation was then shown with purified, antigen-binding normal B cells (12) and also with antigen-specific, virus-transformed B cell lines (13). The polyclonal anti-Ig system was extended to show that small resting B cells respond with a vigorous polyclonal antibody response as a result of presenting antigen to T helper lines or hybridomas (14) and that they can process and present monovalent antigens bound to their antigen receptors (15) while in the resting state (16). In each case, receptor-mediated or antigen-specific antigen uptake and presentation was 100- to 10,000-fold more efficient than nonspecific antigen presentation (13, review). This remarkably efficient presentation of antigen by antigen-specific B cells accounts, at least in part, for the specificity of the antibody response.

Inducible Costimulatory Activities in B Cells

The in vitro experiments mentioned above showed that small B cells are efficient APCs for inducing helper signals from T cell lines and hybridomas, but helper signals can be induced from helper T cell lines or primed T cells without T cell proliferation. Other studies showed that small B cells are defective APCs for inducing primary antibody responses in vivo (17, 18) or T cell proliferative responses in vitro (19, review). Small B cells are also defective APC for the proliferative response of established Th1 lines because they fail to express a membrane-bound costimulatory activity (20, 21) and for Th2 lines, because they fail to make IL–1, which some Th2 lines require in order to respond to their own IL–4 in an autocrine fashion

(22, review). Costimulatory activity can be induced in B cells by activation, although different activation signals may induce different, as yet poorly defined, costimulatory activities. B cell blasts generally stimulate T cell proliferation well, and antigen-specific, proliferating B cells have been implicated in driving T cell expansion in vivo in antigen-primed lymph nodes (7, review). However, B cells are not required for T cell responses in vivo; T cell priming for lymph node proliferative responses is normal in mice with severe combined immunodeficiency that are reconstituted with T cells only (23).

If B cells are defective APC for primary T cell expansion and differentiation to helper function, how does the antibody response get started? In vitro studies discussed below support the concept that helper T cells must proliferate and differentiate from precursor cells to effector cells in order to gain helper function (24). There appears to be a requirement for another kind of APC to initiate the helper T cell arm of the antibody response (17, 18), perhaps a dendritic cell (19) or an adjuvant-activated macrophage (25).

B Cells as Tolerogenic Antigen-Presenting Cells

If resting B cells cannot induce proliferation and differentiation to helper function in resting T cells because of a lack of costimulatory activity, what are the consequences for the virgin T cell of recognizing antigen on a resting B cell? It has been proposed that primary T cells that recognize antigen in the absence of costimulation become functionally inactivated (26). In some Th1 clones, antigen recognition without costimulation results in a stable state of unresponsiveness called clonal anergy (27). Perhaps a virgin T cell becomes functionally inactivated when it encounters antigen on a resting B cell. In fact, transferred B cells have been shown to induce skin graft tolerance to an isolated class II alloantigen (28) and the H-Y minor histocompatibility antigen (Ephraim Fuchs, Polly Matzinger, *Science* 258: 1156–59). A foreign protein antigen targeted to B cells in vivo was a particularly effective tolerogen for antigen-reactive T cells in unprimed animals (29). These experiments support a role for B cells as antigen-specific APCs in acquired tolerance to protein antigens and in self tolerance to self antigens, especially those self antigens present in concentrations too low to induce tolerance through nonspecific antigen presentation by other types of APCs in the thymus, as argued elsewhere (10, review).

B LYMPHOCYTE ACTIVATION STAGES AND SUBSETS

Although the small, resting lymphocyte is poised to respond to external signals by proliferation and differentiation to effector function, the

response is not all-or-none. Lymphocyte activation is a multistep process regulated at each step. As implied above in the discussion of costimulation for T cells, multiple external signals result in an integrated cellular response. Uncovering causal connections among external signals, intracellular signaling pathways, and lymphocyte responses is difficult because the biochemical events associated with signaling pathways occur in minutes or seconds, while the lymphocyte response occurs over a period of days. It is helpful to consider B lymphocyte activation in stages: early events and priming, entry into cell cycle, progression through the cell cycle, and differentiation to Ig secretion and class switching, and to study signals which move the B cells through one stage to the next.

While in vitro B lymphocyte culture systems have been invaluable in defining this activation sequence and the various signals which can drive B cells along it, they have their limitations. B cells can be made to proliferate, secrete Ig, and switch Ig class in vitro, but these cultures are short-lived. With the exception of an anti-CD40-driven system (30), most of the B cells are dead or terminally differentiated to Ig-secreting cells within a week or at most two weeks. Culture systems that will generate long-lived memory B cells or propagatable nontransformed clonal B cell lines, or that undergo somatic mutation or affinity maturation, remain to be developed.

Also, normal B cells are heterogeneous in their ability to respond to activating signals in vitro. In particular, a fraction of freshly prepared B cells has less stringent activation requirements than the major population of small B cells (9), a fact which led some years ago to considerable confusion about whether T/B collaboration is in fact MHC-restricted in vitro (4, review). Subsequently, there has been an emphasis on the study of small, resting B lymphocytes, from which the partially activated cells have been removed on density gradients or by size fractionation using velocity sedimentation or centrifugal elutriation.

There are additional B lymphocyte subsets unrelated to changes accompanying acute activation. The major population in the mouse spleen are small, dense, resting, primary B lymphocytes some of which are recently differentiated from precursors in the bone marrow. Another population of small B cells is long-lived and includes the memory cells produced by clonal expansion during a previous exposure to antigen, although B cells can enter a long-lived compartment without deliberate antigen exposure (31). In addition to these differentiation states of the major population of B lymphocytes, there may be other, distinct B lymphocyte lineages with different life histories and functions. These include the marginal zone B cells, which account for the T cell–independent response to polysaccharide antigens (31) and the $Ly1^+$ ($CD5^+$) B cells in the mouse (32), which form a self renewing population in adults. Although there is little evidence that

$CD5^+$ B cells participate in normal thymus-dependent responses, they have high rates of spontaneous Ig secretion and may account for most of the circulating immunoglobulins and the natural antibody in unimmunized individuals.

Little is known about the extent to which these minor B cell subsets contribute to polyclonal T-dependent responses in vitro. The $CD5^+$ and marginal zone B cells are larger and less dense, and so may be removed when small, resting B cells are prepared. The frequency of resting B lymphocytes which respond to polyclonal T cell help is as high or higher than that responding to other activating signals (33–35). However, when not all the B cells respond, it raises the possibility of further heterogeneity in the major resting B cell population. This possibility becomes important in interpreting experiments in which different activating signals give rise to different outcomes: are the same B cells responding in different ways, or are different B cells responding? Unequivocal answers to questions about the timing of commitment to a particular outcome during B cell differentiation are often difficult to obtain. In the case of immunoglobulin class switching, the weight of evidence is strongly in favor of a pluripotent small B cell that can be driven in different directions by different signals (34–36).

THE ROLE OF SIGNAL 1 THROUGH MEMBRANE IMMUNOGLOBULIN

A key tenet of the clonal selection theory is the selection of clones committed to make particular antibodies by the interaction of antigen with a sample of the antibody displayed as an antigen receptor on the plasma membrane. The fact that B cells could be activated by antibodies specific for membrane Ig was taken as early direct evidence for the clonal selection theory. To accommodate other evidence on acquired tolerance and the requirement for linked recognition of hapten and carrier, Bretscher & Cohn (37) proposed that the B cell required two signals, the first through the antigen receptor, and the second from the carrier-specific helper T cell, which was targeted to the B cell by antigen. Later, based on experiments with haptenated lipopolysaccharide showing that immunogenicity could be a function of the intrinsic activating properties of the antigen, Coutinho & Möller proposed the "one non-specific signal" theory, in which signal 1 through membrane Ig was declared to be irrelevant in T-dependent responses to protein antigens as well (38).

Since then, abundant evidence has accumulated that B cells can be effectively activated through cross-linking of membrane Ig (39–41, reviews). This signal is likely to be essential for the T cell–independent

antibody response to polysaccharide antigens and other such highly multivalent, type 2 thymus-independent antigens, which lack peptide determinants for helper T cells. CBA/N mice bearing the *xid* mutation, which fail to respond to polysaccharide antigens, are also defective in response to membrane Ig cross-linking in vitro (42, 43). High concentrations of $F(ab')_2$ fragments of high affinity anti-IgM antibodies are mitogenic for purified small B cells, but responses can be enhanced and driven on to Ig secretion by provision of appropriate lymphokines. Submitogenic membrane Ig cross-linking can induce responsiveness to IL–4 and other lymphokines (44), and membrane Ig cross-linking upregulates expression of the IL–2 receptor (45), class II MHC (46), adhesion molecules (47), and costimulatory activity (20).

It would be surprising if these effects were irrelevant to T/B collaboration, and indeed, some early papers on polyclonal T/B collaboration established a requirement for signal 1 through membrane Ig in a response that was also dependent on MHC-restricted T cell help (48, 49). Other effects of Ig cross-linking on antigen presentation or T-dependent B cell responses have been reported (50, 51, review). On the other hand, a number of other investigators found excellent, T-dependent, MHC-restricted responses in the apparent absence of any involvement of membrane Ig, using alloreactive T cell lines or loading B cells with antigen nonspecifically at high antigen concentration (8, 9, 50, 52). When help was provided by an antigen-specific Th2 line, we were surprised to find that monovalent antigen was just as effective on a weight basis as divalent, activating antigen for proliferation and Ig secretion by small B cells (15). Moreover, in the same system, addition of a cross-linking, activating monoclonal anti-IgM antibody did not shift the dose response curve of monovalent antigen bound initially to membrane IgD (15).

Why is signal 1 required in some experiments and not others? We would argue that the experiments cited above demonstrate that signal 1 is in fact not necessary for T-dependent B cell activation, and that an appropriately activated T cell can provide all the necessary signals for B cell proliferation, Ig secretion, and class switching without a signal through membrane Ig. In the experiments in which a signal through membrane Ig is required, it is possible that the T cells employed could not deliver the full range of helper signals. For instance, as discussed further below, Th1 T cell lines are generally poor helpers in vitro but can be reconstituted with Th2 lymphokines. Signal 1 through the antigen receptor could induce high affinity IL–2 receptors or otherwise enable B cells to respond to insufficient helper signals. Alternatively, signal 1 may be necessary to allow the B cells to interact effectively with some T cells in order to induce the required helper signals, for instance, to induce costimulatory signals or to enhance

antigen presentation by increasing class II expression, adherence, or alloantigen expression.

It is not unlikely that signal 1, even when unnecessary for a vigorous antibody response, could be integrated along with contact help, cytokines, and other signals to affect the quality of the antibody response. For instance, anti-Ig strongly suppresses differentiation to high rate Ig secretion in the LPS response (53), and it may also influence switching to particular isotypes by effects on germline constant region transcription (54, 55).

The question of the role of signal 1 is harder to establish in vivo. Initially monovalent antigen can become multivalent by binding to cell surfaces directly or through antibody and Fc receptors or complement receptors. In addition to enhancing antigen presentation and costimulation as described above, signal 1 could play additional roles in vivo such as the preferential localization of antigen-specific B cells in appropriate microenvironments to meet antigen-specific T cells through enhanced adherence or chemotaxis, or selection of rare, high affinity somatic mutants from the progeny of germinal centers through direct signaling by antigen through antigen receptors. Anti-Ig cooperates with anti-CD40 to prevent apoptosis in germinal center centrocytes (56). A requirement for membrane IgD cross-linking was demonstrated in a model for a primary, T-dependent antibody response, the in vivo polyclonal IgG1 antibody response to anti-IgD antibodies (57).

CONTACT-DEPENDENT HELP

The Need for Contact-Dependent Help

No combination of characterized lymphokines induces clonal expansion in carefully prepared small resting B cells; there appear to be necessary short-range or contact-dependent signals delivered to the B cell when it presents antigen to the T cell (58, 59, reviews). This has been shown both indirectly by MHC restriction of T/B collaboration in specific antibody responses and directly in T-dependent polyclonal B cell responses. Soluble T cell–derived factors which have been reported to induce proliferation and differentiation of resting B cells have resisted isolation and molecular identification (60). Although B cells can be made responsive to lymphokines through strong cross-linking of membrane Ig by anti-Ig (40) or polysaccharide antigens (61), a signal through the antigen receptor is not required for a vigorous T cell–dependent B cell response as explained above, and the degree of cross-linking of membrane Ig which follows binding of soluble protein antigens to the B cell fails to induce responsiveness to lymphokines (62, 63).

Models of Contact-Dependent Help

One model for short-range interactions of T cells with antigen presenting cells is the killing of target cells by cytolytic T cells, which usually spare bystander cells. Although the mechanisms of killing are redundant and controversial, one mechanism involves directional secretion of pre-formed mediators by degranulation across the small intracellular space between the lymphocyte and the target cell (64). Helper factors have not been reported to be packaged in granules in helper T cells, but B cells might receive very high levels of stable lymphokines or postulated labile lymphokines by directional secretion of newly synthesized mediators. Helper T cells form tight conjugates with B cells (65), and there is functional (66) and microscopic (67) evidence for directional secretion of lymphokines toward the antigen-presenting B cell.

Other models for the delivery of contact help require direct interaction of membrane-bound molecules. That is, after all, how the T cell gets its activating signal through its antigen receptor and CD4 and the other accessory molecules involved in antigen recognition. It seems natural that the B cell should get simultaneous contact-dependent signals through its set of ligands for the molecules that activate the helper T cell. These include class II MHC (the ligand for the T cell antigen receptor, and CD4) in addition to the adhesion molecules like the LFA–1/ICAM–1 and CD2/LFA–3 ligand pairs, which also have signaling functions (68). In this model, B cell activation is a direct consequence of T cell recognition of antigen on the B cell surface.

Inducible Contact Help in Noncognate Systems

However, contact-dependent B cell activation can be divorced from antigen recognition in certain experimental systems in which the T cell is activated independently of antigen presentation by the responding B cell. This can be done by studying polyclonal "bystander" or "noncognate" responses of B cells that lack the appropriate MHC alleles or are not presenting antigen. In these systems, T cells are activated by mitomycin C–treated antigen-presenting B cells or plate-bound or cell-bound anti-CD3 or anti-T cell receptor antibodies. These bystander and noncognate responses are intense in the hands of many investigators, showing that antigen recognition can be separated functionally from the delivery of help (50, 69–76). For resting B cell responses, cell contact is required (72, 73, 75). These systems imply that some limiting component of the delivery of bystander or noncognate help requires activation of the T cell. In this model, activation is sequential rather than simultaneous: the B cell must first activate the T cell before it can receive activating signals from the T

cell. We have called this induced activity of helper T cells ITCH, for inducible T-dependent contact help (77).

More direct evidence for sequential rather than simultaneous activation of T and B cells during T/B conjugation was provided by Brian when she showed that plasma membranes from activated but not resting T cells could deliver contact-dependent help to B cells (78), a finding that has been reproduced and extended by others (79, 80). Similarly, Noelle and colleagues showed that activated T cells retain contact-dependent helper activity when they are metabolically inactivated by treatment with paraformaldehyde (81). This finding has also been reproduced and extended (77, 82). Fixed resting T cells and plasma membranes from resting T cells are not active. The acquisition of helper activity is transient and requires several hours of activation (59, review, 77). Acquisition of helper activity is sensitive to cyclosporin A (CsA) (79, 82, 83) and inhibitors of RNA and protein synthesis (59, 79, 82).

The contact helper activity of fixed cells and activated membranes also shows that directional secretion of pre-formed or newly synthesized helper factors is not an essential component of contact-dependent help, because the fixed cells and membranes are no longer capable of secretion, directional or otherwise, and retain activity during storage for several weeks at 4°. Induction of contact help requires a change in the helper T cell surface. The change could involve a modification of an existing membrane structure, for instance, the rapid increase in affinity of LFA–1 for ICAM–1 following T cell activation (84). However, the time course of induction of helper activity and the sensitivity to CsA and other drugs strongly suggests a requirement for synthesis of a new protein from a gene regulated like a lymphokine by T cell activation. Therefore, the limiting component of help in the noncognate system appears to be one or more membrane-bound lymphokines or transient T cell activation antigens that act by engaging receptors on B cells. The recently identified ligand for CD40 on activated helper T cells is an excellent candidate for such a molecule (85, 86) and is discussed below under its own heading.

Early Signals in Contact Help Depend on T Cell Activation

Although the non-cognate systems provide strong evidence for sequential activation of T cells and B cells during T/B collaboration, it can be argued that contact help is likely to be complex and redundant, and that other signaling pathways could be used in cognate interactions that result in simultaneous activation of the T cell and the B cell as a direct result of T cell recognition of antigen presented by the B cell. At limiting antigen concentrations and in some systems in which antigen concentration cannot be varied, bystander contact help is weak or absent (8, 52, 87). Even in

systems in which bystander help is easy to demonstrate, cognate help is more efficient than bystander help, especially when one measures Ig secretion rather than DNA synthesis in the responding B cells (14, 50, 70, 72). In a "cold target inhibition" experiment, mitomycin C-treated antigen-presenting B cells but not mitomycin C-treated bystander B cells compete with untreated B cells for limiting contact-dependent help signals (72). It has been argued that bystander help requires maximal, nonphysiological levels of helper T cell activation. To test whether cognate help involves growth signals delivered to the B cell as a direct consequence of Ag-specific conjugation, or whether the B cell must wait for some activation-induced change in the T cell in order to get its contact-dependent helper signal, one needs an early measure of the receipt of growth signals by the B cell.

To look as early as possible in the B cell activation sequence during T cell/B cell collaboration, we developed quantitative assays for the expression of the early activation genes c--*myc* and *egr*–1 during antigen specific T/B interaction (83). In our experiments, resting B cells are pulsed with monovalent antigen overnight and then mixed at high cell concentrations with helper T cells. After various periods of interaction at 37°, T and B cells are separated at 0° with antibody-coated magnetic beads, and RNA levels are analyzed with a probe protection assay.

The T cell–dependent, antigen-dependent c-*myc* signal in the B cells begins at 2 hr and peaks at 4 or 8 hr after T and B cell are mixed. We initially interpreted this rapid B cell response as evidence that growth signals can be delivered to the B cell without a requirement for lymphokine synthesis or induction of other helper machinery that needs to be made from new mRNA in the T cell. However, the same very early c-*myc* induction in B cells occurs when B cells receive help from T cells activated by plate-bound anti-CD3 without antigen. Also, the B cell c-*myc* response is sensitive to CsA acting on the T cells rather than the B cells. Therefore, this earliest growth response of the B cell in T/B conjugates also appears to require an inducible activity in the T cell (83). By this sensitive assay for growth signals, we found no indication of a B cell growth response triggered solely by the preformed membrane molecules involved in adhesion and T cell Ag recognition.

Early and Late Components of Contact Help

Although small B cells can receive growth signals as soon as 2 hr after conjugation with cloned helper T cells, it takes at least 6 to 8 hr of activation for T cells to gain the ability to induce DNA synthesis in B cells, when T cell activation is stopped by addition of paraformaldehyde or CsA, and excess IL–4 is provided (77, 88). The ligand for CD40, which is likely to be a key component of inducible contact help, is expressed very rapidly

on cloned T cell lines and may account for early contact help, but levels of CD40-ligand expression reach a peak at 3 or 4 hr of activation and begin to decline while helper activity of plasma membranes prepared from the same cells continues to increase (B. E. Castle, K. Kishimoto, M. L. Brown, M. R. Kehry, personal communication). Therefore, as mentioned above, there may well be multiple components of inducible contact help, some of which are expressed very early in the T cell activation sequence, and some of which are expressed later.

Requirements for Induction of Contact-Help Activity

Induction of contact help activity in continuous T cell lines follows the same time course as lymphokine synthesis and release, and it is likely to be regulated by the same intracellular signaling pathways originating from the T cell antigen receptor and the receptors for costimulatory signals on the T cell surface. Th1 and Th2 murine T cell lines show important differences in signaling pathways leading to lymphokine secretion (22, 89). The same differences, as revealed by sensitivity to various drugs that block intracellular signaling, apply to induction of contact help activity (77).

Contact help, like IL–4 production, is difficult to induce by polyclonal activation in resting normal T cells from healthy mice. Expression of these activities of differentiated helper T cells requires activation and culture for several days, followed by restimulation (24, 87, 90) (B. Whalen, D. C. Parker, unpublished). Therefore, it seems that precursors of helper T cells, like precursors of cytolytic T cells, must proliferate and differentiate to effector cells before they can express their differentiated functions efficiently (24). T memory helper cells from primed and rested animals, like in vivo primed cytolytic T cells, may also require a recent boost in vivo or in vitro to differentiate into effector cells (24), although in vivo T helper function in primed animals is generally radioresistant. Contact help can be induced directly in normal human peripheral blood T cells (82), but this population may include T cells mobilized by recent antigen exposure and primed for helper function.

THE ROLES OF CYTOKINES

The activity in supernatants of activated T cells originally called "T cell replacing factor" has turned out to be owing to a complex and variable mixture of cytokines. T cell replacing factor was resolved into various B cell growth and B cell differentiation factors by the early 1980s, but it was not until the availability of recombinant cytokines and neutralizing monoclonal antibodies that definitive experiments could be done. It is now

clear that the various cytokines involved in antibody responses in vitro, including IL–2, IL–4, IL–5, IL–6, IL–10, and γ-interferon, act in various combinations at various stages of the complex B cell activation sequence (91, 92). Different cytokines can produce similar effects, and the same cytokine can have different effects early and late in the activation sequence, depending also on the presence of other cytokines or signals. None is either necessary or sufficient for B cell growth or differentiation.

Th1 and Th2 Cells

The best insights into the roles of cytokines in T-dependent antibody responses have come from the study of helper activities of the Th1 and Th2 T cell lines in the mouse, which are distinguished by their stable patterns of lymphokine expression (36). Th1 cells secrete IL–2, γ-interferon, and lymphotoxin but not IL–4, IL–5, or IL–10, while Th2 lines secrete IL–4, IL–5, and IL–10 but not IL–2, γ-interferon, or lymphotoxin.

Both Th1 and Th2 cell lines are effective helper cells in vivo, including the generation of B cell memory and affinity maturation (93). Most Th2 cell lines are more effective helper cells than most Th1 lines for resting B cells in vitro (94), particularly for polyclonal responses (22, review), but Th1 cells help well if γ-interferon levels are low and cultures are supplemented with Th2 lymphokines (36), or if B cells belong to a subset expressing relatively high levels of class II antigen that is found in conventionally housed mice but not in sterilely housed mice (95). Therefore, both kinds of T cells deliver contact help signals, and antibody responses can be driven with either Th1 or Th2 lymphokines, although the requirements for induction of functional, high affinity IL–2 receptors on resting mouse B cells and their role in Th1-dependent responses have not been fully defined (J. Poudrier, T. Owens, personal communication).

A consistent and important difference between B cell antibody responses driven by Th1 and Th2 cell lines is in the classes of antibodies secreted by the responding B cells. Th1 helper cells preferentially induce IgG2a, while Th2 helpers induce IgG1 and IgE secretion (36, review). This difference appears to be owing entirely to the different cytokines produced, which direct switching by activating transcription of Ig constant region genes in their germline configurations (96). IL–4 enhances transcription of IgG1 and IgE germline constant region genes, and IL–4 is required for switching to IgE in vivo and in vitro (36). γ-interferon inhibits IgG1 and enhances IgG2a secretion. Contact helper signals delivered by membranes from activated Th1 and Th2 cells are equivalent (80, 97), although contact signals themselves may influence transcription of Ig heavy chain constant region germline genes and hence switching (98).

Lymphokines and Contact Help

Although some laboratories can detect a B cell proliferative response to contact help provided by activated and fixed T cells alone (77) or activated T cell membranes alone (79), these responses can be greatly enhanced by the addition of exogenous lymphokines (99, review). In the mouse, proliferation is enhanced by IL–4, and IL–4 accounts for all the proliferation enhancing activity in activated Th2 culture supernatant (80, and K. Kawakami, D. C. Parker, unpublished). Secretion of Ig and class switching depends on addition of IL–4 and IL–5 (80, 97). For human B cell responses to activated and fixed T cells, IL–2 plays a key role, although IL–4 and IL–6 can enhance Ig secretion (82). IL–4 is required for the human IgE response to contact with activated and fixed T cells (100). IL–4 also plays a unique role in the human B cell proliferative response and IgE response to anti-CD40 antibody (30, 101), and it is required for IgE secretion of mouse and human B cells in response to transfected cells expressing CD40-ligand, although the transfected cells induce B cell proliferation by themselves (85).

INTRACELLULAR SIGNALING PATHWAYS IN CONTACT HELP

Not much is known about intracellular signaling pathways in B cells in response to contact help. Strong induction of inositol phospholipid turnover and a rapid and transient increase in intracellular calcium ion concentration have been recently reported in a human B lymphoblastoid line presenting antigen to a human T cell line (102), but these signals had been sought unsuccessfully in normal human B cells receiving help from T cells (103). Changes in intracellular calcium ion concentration were also sought but not found in normal mouse B cells forming conjugates with anti-CD3 activated T cells (K. Kawakami, D. C. Parker, unpublished). A very rapid increase in cAMP concentration in B cells interacting with fixed, activated T cells has been reported (104). This increase could not be detected using activated plasma membranes (R. Noelle, personal communication). Early effects of contact help within 2 to 4 hr include induction of c-*myc* expression, as discussed above (83), and also appearance of a DNA binding protein in the NF-κB transcription factor family (A.-C. Lalmanach-Girard, T. C. Chiles, D. C. Parker, T. L. Rothstein, unpublished). Later effects include induction of ornithine decarboxylase activity (104) and casein kinase II activity (105), an increase in expression of CD23 (103, 106) and class II, and the increase in cellular size and RNA content that accompanies entry into G1 (81). Contact with T cells induces changes in

various other membrane proteins (107, and H. Wortis and D. C. Parker, unpublished). Activated T cell membranes induce transcripts from the IgG1 constant region (98).

A number of drugs block activation of B cells through membrane Ig cross-linking, including CsA, phorbol esters added at the time of activation, overnight incubation in phorbol ester to deplete PKC activity, and agents that raise cAMP. The same drugs do not block activation of B cells by contact with fixed, activated T cells, or block only at hundred-fold higher concentrations, as measured by DNA synthesis in the presence of added IL–4 (107a). This finding, and the lack of an obvious calcium and phosphatidyl inositol signal in small B cells, suggests that T cells use a signaling pathway that is distinct from that engaged through membrane Ig. Also, in the early activation gene experiments mentioned above (83), contact with T cells in either cognate or noncognate conjugates caused a large increase in c-*myc* mRNA levels without the corresponding increase in *egr*–1 levels that accompanies B cell activation by anti-Ig (108).

Contact help may involve a molecular interaction between a T cell membrane molecule and the membrane Ig complex. For instance, two laboratories have reported that CD4 associates directly with Ig (109, 110). Also, T cells bear an inducible Fc receptor for IgD (111). Alternatively, an inducible membrane protein on T cells might interact and cross-link membrane Ig or another molecule, such as CD21 (47, for references), associated with the membrane Ig complex. However, we think these possibilities are made unlikely by the experiments showing that the signaling pathways involved in contact help appear to be different from those engaged by cross-linking membrane Ig.

Progress on signaling pathways in contact help will be furthered by identification of the molecules involved in delivering help to the B cell across the B cell plasma membrane. With the appropriate reagents, signal transduction can then be studied following optimal cross-linking of individual molecules by monoclonal antibodies or soluble ligands or cell lines transfected with genes encoding membrane-bound ligands, as can now be done with CD40 and its ligand, a process described below.

MOLECULES INVOLVED IN DELIVERY OF CONTACT HELP

The number of ligand pairs involved in the T/B interaction is surprisingly large and growing. A partial list of ligand pairs and other surface molecules seeking ligands is shown in Table 1. Many of these molecules are known to be involved in T cell recognition of antigen on the B cell surface, either directly or as adhesion/signaling accessory molecules. Others act as

Table 1 Ligand pairs in T/B interaction

	T cell	B cell	Reference
Antigen recognition	TCR/CD3/CD4	Class II/peptide	158
	CD4	Class II	129
Adhesion/signaling	LFA-1	ICAM-1	68, 102, 137
	ICAM-1	LFA-1	138
	CD2	LFA-3	141
Costimulation	CD28	B7	144, 148
	CTLA-4	B7	150
	?	Heat stable antigen	159
Inducible contact help	CD40-ligand	CD40	85, 86
New ligand pairs	CD45Ro	CD22	146
	CD5	CD72 (Lyb-2)	145
	LAG-3	Class II	132
TNF receptor family	OX40	?	117
	CD27	?	122
	4-1BB	?	123
	Membrane TNF?	TNF receptor	118
Unknown	Fcδ receptor	IgD?	111
	CD4	Ig?	109, 110
	?	CD19	47
	?	CD20	47
	?	Bgp95	47

costimulatory molecules to modify the T cell response to antigen recognition. As argued above, we think it is unlikely that these molecules directly deliver essential, simultaneous, early growth signals to the B cell. On the other hand, to the extent that the B cell partners of these ligand pairs have been shown to act as signal transducing molecules, they are likely to modify either early signals or an ongoing interaction. From the point of view of a minimal model of contact help, the two most interesting classes of molecules are the activation antigens that appear rapidly on the T cell surface and the resident surface antigens on B cells that can activate B cells when cross-linked with monoclonal antibodies, including CD72 (Lyb2), CD40, CD19, CD20, and Bgp95 (47).

CD40-Ligand

At least five laboratories have independently identified what may be a single new surface protein, restricted in its expression to acutely activated $CD4^+$ T cells, that binds the B cell differentiation antigen, CD40, and activates B cells (85, 86; B. E. Castle, K. Kishimoto, M. L. Brown, M. R. Kehry, personal communication; S. Lederman, M. J. Yellin, G. Inghirami,

J. J. Lee, D. M. Knowles, L. Chess, *J. Immunol.* 149: 3817–26; P. Lane, A. Traunecker, S. Hubele, S. Inui, A. Lanzavecchia, D. Gray, *Eur. J. Immunol.* 22: 2573–78). Using a soluble CD40-Ig fusion protein, Armitage and colleagues (85) identified and cloned a CD40 ligand (CD40L) from a cDNA library made from a variant of the EL4 murine thymoma line, which they had selected for high expression of binding activity for soluble CD40-Ig. The cDNA encodes a predicted type II membrane protein of Mr about 33,000, with homology to tumor necrosis factor-α (TNF-α) and lymphotoxin (LT, also called TNF-β) (112). Expression of the message is strongly and rapidly induced in murine T cell lines by activation. A cell line transfected with the CD40-ligand clone induces proliferation and, in the presence of IL–4, IgE secretion from murine and human B cells. Noelle and colleagues (86) have isolated a hamster monoclonal antibody specific for an activation antigen on a murine T helper line that cross-competes with a soluble CD40-Ig fusion protein in binding to activated T helper cells, and that precipitates a protein of the same size as CD40-ligand. Both the antibody and the CD40-Ig molecule block the helper activity of membranes from activated T helper cells. Kehry and colleagues (B. E. Castle, K. Kishimoto, M. L. Brown, M. R. Kehry, personal communication) and Lane and colleagues (P. Lane, A. Traunecker, S. Hubele, S. Inui, A. Lanzavecchia, D. Gray, personal communication) each have data on the expression and helper function of CD40-ligand based on experiments with soluble CD40 molecules, in mouse and human systems, respectively. Lederman and colleagues have a monoclonal antibody specific for a variant subclone of the Jurkat human leukemic line which exhibits constitutive contact help activity (106). The help activity is blocked by a nonstimulatory form of anti-CD40. The monoclonal antibody detects a transient activation antigen of normal $CD4^+$ human T cells and inhibits the contact help activity of activated and fixed T cells. It stains rare $CD4^+$ T cells localized in the mantle and centrocytic zones of lymphoid follicles (S. Lederman, M. J. Yellin, G. Inghirami, J. J. Lee, D. M. Knowles, L. Chess, personal communication) and is likely to react with CD40-ligand or a very similar molecule.

The identification of CD40-ligand as a component of contact help will focus interest on CD40 as a signal transducing molecule. Anti-CD40 has recently been reported to induce c-*myc* expression (113). Antibodies to CD40 and several other B cell surface molecules upregulate cell adhesion through LFA–1 and other adhesion molecules (114). Anti-CD40 antibodies costimulate B cell proliferation together with IL–4, anti-Ig, or anti-CD20 antibodies (47, review). Cross-linking of CD40 induces rapid and extensive phosphorylation of CD20, and in the buoyant, activated tonsillar B cell fraction causes increases in phosphotyrosine and serine/threonine

kinase activity comparable to those produced by anti-Ig or anti-class II antibodies (115). Anti-CD40 antibody and IL–6, the latter by an indirect effect dependent on CD40, inhibit growth of CD40-transfected B cell lines (116).

Other Members of the CD40/TNF Receptor Family

CD40 is a member of a new class of membrane receptor proteins (117) which includes the low affinity nerve growth factor (NGF) receptor and the two TNF receptors for TNF-α and LT. TNF-α appears in Table 1 because it is a cytokine that has been reported to occur in an active, membrane-bound form (118), and it and LT, a Th1 lymphokine, act on the same receptors and are stimulatory for B cells (119). However, we were unable to inhibit contact help activity (C. Zarozinski, D. C. Parker, unpublished) with a soluble TNF receptor that blocks both TNF-α and LT activity (120). Another member of the receptor family is the Fas antigen, thought to be a trigger for apoptosis in thymocytes and other cell types (121). Three other members of this receptor family appear in Table 1 because they are T cell activation antigens: OX40 (117), CD27 (122), and the activation gene, 4–1BB (123). Of these, OX40 is of particular interest because its expression is restricted to $CD4^+$ T cells (117). However, since these molecules are homologous to receptors rather than their ligands, they probably costimulate or regulate helper T cell activation, rather than directly activate B cells.

Class II MHC

Because it is the B cell contribution to the ligand pair that is required for specific antigen recognition by the T cell, the class II MHC molecule is a favorite candidate for receipt of helper signals when the B cell presents antigen to the T cell. Class II MHC molecules are known to signal B cells by various pathways, and anti-class II antibodies have been frequently reported to have stimulatory or inhibitory effects on B lymphocyte responses (124–127). In combination with suboptimal stimulation with anti-Ig or anti-Ig and IL–4, anti-class II antibodies can induce B cell proliferation (127, 128).

Antibodies to class II molecules effectively block cognate help by blocking T cell antigen recognition. To study effects of anti-class II antibodies or other antibodies on the delivery rather than the induction of help, it is necessary to use bystander or noncognate systems. Of course, the noncognate systems by themselves argue against a role for an antigen-specific, MHC-restricted class II interaction in B cell activation, since the bystander and noncognate responses are not MHC-restricted. But it is known that class II can be engaged directly by CD4 (129), and CD4 redistributes to

the area of cell contact in T/B conjugates (130), and it has been argued that this interaction or a nonpolymorphic interaction with some portion of the T cell antigen receptor complex could participate directly in the delivery of noncognate help (81, 131). Alternatively, some component of inducible contact help on the T cell surface could engage class II; LAG–3 is a T cell activation gene which encodes a protein of unknown function that is closely related to CD4 and binds class II (132). Indeed, one could argue that effective signaling through class II in cognate systems should not be limited to the relatively rare interactions of a T cell antigen receptor with a particular class II peptide complex, of which only a few hundred may be adequate to trigger the T cell response (133).

The effects of anti-class II antibodies on noncognate responses have been mixed. Anti-CD4 or anti-class II antibodies reportedly can block delivery of help in noncognate systems by blocking a CD4/class II interaction (81, 134). We found that anti-class II antibodies against bystander alleles of class II do not block the bystander response (72). Others have also found that anti-CD4 or anti-class II antibodies fail to inhibit noncognate help from activated T cells, T cell membranes, or killed activated T cells (79, 86, 135). Looking very early in the activation sequence, we showed that anti-class II antibodies which block antigen recognition do not block the early c-*myc* signal in B cells induced by fixed, activated helper T cells (83). The possibility of negative signaling through class II could explain the apparent blocking effect of anti-class II antibodies in some experiments. Alternatively, the inability to block could be explained by proposing that the anti-class II antibodies replace the contact help signal from the T cell.

Recently, the availability of "knockout" mice lacking both I-A and I-E molecules produced by homologous recombination in embryonic stem cell lines (136) has allowed us to test unequivocally the requirement for class II molecules in the delivery of contact help in the noncognate model. We found that class II negative mice respond as well as class II positive heterozygous littermates by DNA synthesis and immunoglobulin secretion of various isotypes to Th1 or Th2 cells activated by plate-bound anti-CD3 (136a). Another laboratory has shown that MHC class II-negative variant B cells respond as well as class II-positive B cells in the rapid calcium and inositol phospholipid response of human B cell lines to contact with activated T cell clones (102). Therefore, class II molecules appear to play no essential role in the effector phase of help once the helper function is induced, at least in the noncognate models.

Adhesion/Signaling Molecules

In addition to engagement of the T cell antigen receptor and CD4 with the class II peptide complex, successful antigen recognition frequently

requires the participation of additional signaling/adhesion ligand pairs, of which LFA–1/ICAM–1 or ICAM–2 and CD–2/LFA–3 are the best studied (68). These interactions could also aid in the delivery of help to B cells, either as adhesion molecules to enhance delivery of signals by other molecules or as signaling molecules themselves. As discussed above for class II molecules, the role of these additional ligand pairs in the delivery of help is difficult to study in cognate systems because they are also required for the induction of help. For instance, in cognate or bystander interactions, anti-LFA–1 antibodies block B cell responses effectively by blocking antigen recognition and T cell activation (72). In various noncognate systems, the effects of antibodies to LFA–1 and ICAM–1 are again mixed, with several groups reporting no effect on B cell responses to activated T cell membranes (86, 99, 134). With anti-CD3-activated T cells or fixed, activated T cells, there is consistent evidence that the LFA–1/ICAM–1 interaction at least enhances the delivery of help (82, 135, 137, 138).

LFA–1 and ICAM–1 can be expressed on both T and B cells. Using T cell lines derived from a person whose T cells lack surface expression of LFA–1, one group (138) showed that LFA–1 on the T cell is not required for the delivery of help in a human noncognate system, and they suggested that ICAM–1 on the T cell engages LFA–1 on the B cell in the delivery of help. ICAM–1 is an activation antigen that is upregulated upon T cell activation (68). ICAM–1 has been shown to have a signaling role through engaging LFA–1 as well as an adhesion function in both T cells and B cells (102, 139, and references therein). A particular, atypical antibody to murine LFA–1 mimics the effects of IL–4 on B cells by binding to LFA–1 (140). Perhaps this antibody mimics the effects of ICAM–1 on B cells. Like IL–4, ICAM–1 on the T cell might deliver progression signals to CD40-ligand-activated B cells. However, another group concluded that a rapid, antigen-specific signal from a cloned T cell to an antigen-presenting B cell line required LFA–1 on the T cell and ICAM–1 on the B cell (102).

CD2 on the T cell has also been proposed to act in the delivery of help (141), although effects of CD2 on induction rather than delivery of help were not completely excluded. Several other groups have reported no effect of anti-CD2 on delivery of noncognate contact help (82, 134, 135). In the mouse, CD2 is expressed on B cells as well as T cells (142), and so it could be a target of an activating interaction. The murine homolog of the ligand for CD2, LFA–3, has not yet been identified. An additional ligand for CD2 in humans, CD59, has been identified recently (143).

Recently Identified Ligand Pairs

Using cDNA clones expressed as membrane proteins or as soluble fragments or fusion proteins, a number of new interactions have been found

among known T and B cell surface antigens. In addition to the CD40-ligand/CD40 interaction, newly identified interactions between T and B cell membrane molecules include CD28/B7 (144), CD5/CD72 (Lyb–2 in the mouse) (145), and CD45Ro/CD22 (146).

The CD28/B7 interaction is a key costimulatory signal for T cell IL–2 production and proliferation (144) and can determine whether antigen presentation results in T cell anergy or proliferation in Th1 lines (147). B7 is upregulated on activated B cells (148, 149) and so may determine in part whether B cells induce tolerance or drive T cell clonal expansion. CTLA–4 is T cell membrane protein which is also a ligand for B7. It is homologous and closely linked to CD28. It is expressed at lower levels but is of higher affinity for B7 than CD28 and was identified initially as a T cell activation gene (150, and references therein). At present, B7 has not been shown to deliver a signal to the B cell (149).

CD72 is a type II integral membrane protein on B cells whose external domain is homologous to that of asialoglycoprotein receptors and CD23. Antibodies to CD72 are mitogenic for murine and human B cells alone or in combination with cytokines. Its ligand, CD5, is expressed on all mature T cells and a subset of B cells, and CD5 can deliver activating signals to T cells when cross-linked with monoclonal antibodies (145, for references). Whether this interaction is costimulatory for T cells or B cells or both in T/B collaboration remains to be determined.

CD22, a marker of mature B cells, is a member of the Ig family and is homologous to myelin-associated glycoprotein. Antibodies to CD22 enhance responses to anti-Ig (47, review). CD45 is the major protein tyrosine phosphatase of lymphocytes and probably plays a key role in T and B lymphocyte activation through the antigen receptors. The CD45Ro isoform is a marker of $CD4^+$ memory T cells in humans, is the dominant CD45 isoform in murine T helper lines, and is upregulated further by acute activation (151, and references therein). A role for this interaction in T/B interactions remains to be established.

Other B Cell Surface Molecules Seeking Ligands

Several other B cell differentiation antigens of unknown function are candidates for receptors for contact help, because antibodies against them have various activating effects. Certain monoclonal antibodies to CD20, a membrane protein with four transmembrane domains used as a pan B cell marker, can activate resting B cells cooperatively with anti-CD40 (47, review, 113), and induce c-*myc* expression by a pathway distinct from that used by mIg (152). As mentioned earlier, anti-CD40 causes extensive phosphorylation of CD20 on serine and threonine. Antibodies to CD19, a member of the Ig family, and Bgp95, an uncloned B cell differentiation

antigen, each have activating effects similar in many respects to those of anti-Ig antibodies, but anti-CD19 antibodies inhibit rather than stimulate c-*myc* expression (47, review).

CONCLUDING REMARKS

The accumulating indirect evidence that MHC-restricted, cell contact–mediated T cell help depends on transient expression of new membrane molecules on helper T cells has been confirmed by the identification of CD40-ligand, which fits exactly the predicted properties of an effector molecule responsible for inducible contact help activity. Contact help has entered the molecular arena, and signal theory in T/B collaboration is about to give way to the study of signaling pathways. Other molecular interactions of known and yet to be discovered ligand pairs will play important roles in the induction and delivery of contact help. This review concentrates on the initial activating signals to resting B cells, but T and B cells continue to interact as proliferating lymphoblasts, and contact signals may be required for later rounds of B cell proliferation (153–155). Unlike CTL, which kill their targets by release of pre-formed mediators and recycle rapidly to kill again, helper T cells engage in a prolonged, perhaps monogamous (156) relationship with an antigen-specific B cell, and there is time for an extended, ongoing dialog in which each cell monitors and influences the activation state of the other. Membrane bound effector molecules are also likely to provide short-range signals to other cells that present antigen to $CD4^+$ T cells, such as macrophages, dendritic cells, and endothelial cells.

The idea that B cells get contact-dependent help by presenting antigen to T cells gave rise to the expectation that the class II molecules themselves would deliver critical activating signals in MHC-restricted T/B collaboration. So far, although anti-class II antibodies signal B cells, there is no good evidence for T cells acting through class II to deliver help. If only the induction but not the delivery of contact help is antigen-specific and MHC-restricted, how is the specificity of T/B collaboration maintained? The effector molecules for contact help could be among those that accumulate in the area of contact between the T cell and the B cell (1, review, 130), and so would not be available to bystanders unless produced in excess. In general, MHC-unrestricted contact help is easier to demonstrate in anti-CD3-driven systems than in bystander systems in which APCs are present. Also, lymphokine-mediated and contact-mediated bystander help have rarely been demonstrated in vivo, where effector mechanisms are tightly regulated and act against a background of nonspecific inhibition.

Membrane-bound effector molecules are more difficult to study than

soluble mediators, but new methods are being developed to identify them and study their functions separately and in combination. Also, the ability to produce mice with homozygous mutations in any cloned gene has created new opportunities to study the function of individual molecules in whole animals, as well as a source of normal cells lacking particular molecules for in vitro studies. In the next few years, knockout mice lacking a variety of cell surface antigens will become available for in vivo and in vitro studies of T/B collaboration.

As part of a larger program of identifying the costimulatory signals which enable T cells to distinguish self from not-self in the periphery (157), future work on T/B collaboration will also investigate how B cells acting as APCs can regulate T cells in immunity and tolerance. On the horizon are useful in vitro systems for reproducing the T cell–dependent germinal center environment, which gives rise to memory cells and in which somatic mutation and affinity maturation occur. More precise definition of cellular interactions, membrane molecules, and activation states in vitro will allow a more incisive approach to the old questions of functional subsets of primary and memory T and B lymphocytes in vivo, the lineage relationships among those subsets, how the rules for cell interactions change following exposure to antigen, and how the system breaks down in autoimmune disease.

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IMMUNOGLOBULIN CLASS SWITCH RECOMBINATION

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KEY WORDS: looping out and deletion, sister chromatid exchange, recombination between homologs, alternative splicing, switch recombinations

Abstract

A B lymphocyte that produces the immunoglobulin heavy (H) chain μ may switch to the production of another heavy chain class: γ, ε, or α. Since the new heavy chain retains the original variable (V) region, antigenic specificity is maintained. The switch is accompanied by a large deletion of DNA at the heavy chain locus. To explain how this deletion is generated, three models have been proposed: recombination between homologs, unequal sister chromatid exchange, and looping out and deletion. While none of the predicted recombination products of the first two models have been found, both by-products of looping out—inversions and circular DNA—have been isolated. Thus looping out and deletion appears to be the appropriate model to explain the genetic events leading to the immunoglobulin heavy chain class switch. One requirement for switching may be transcription of the constant (C) region to which the cell switches. The switch rearrangement is catalyzed by a switch recombinase, and the isolation of the components of this putative enzyme system is in progress. Although the switch deletion is an accepted fact, the discussion is enlivened by scenarios for switching without DNA rearrangement; such suggestions include processing at the RNA level and trans-splicing.

INTRODUCTION

According to Karl Popper (1), a scientific theory cannot be verified; it can only be falsified. This view devalues "positive" evidence for a model and

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stresses the virtues of rejecting a model. But science is a social enterprise (2), and rejecting a model does not win one any friends unless the model is one's own, while positive evidence need not make one any enemies. Furthermore, it is more gratifying to legitimize one model than to banish others. In this review we explore both routes for selecting a model to explain the events in the genome of a B lymphocyte that result in a switch of the immunoglobulin heavy chain class produced by that cell.

In the mouse, the early immune response is dominated by the expression of IgM, which contains the heavy (H) chain μ; later there appear IgG_3, IgG_1, IgG_{2b}, IgG_{2a}, IgA, and (rarely) IgE, which contain the H chains $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, α, and ε, respectively. The organization of the C gene segments at the H chain locus is shown in Figure 1 (3). Except for $C\delta$, each is preceded by a switch (S) region of several kilobases within which the breakpoints for the switch rearrangement are usually found.

Generally, the switch takes place after a committed B lymphocyte has been stimulated by antigen or mitogen (4–8). But a priori, cells may switch from μ to other isotypes, as soon as μ chain is synthesized, i.e. as early as the pre-B cell stage, before light chain is produced. Indeed, Abelson virus–transformed pre-B cells undergo class switching (9); in these cells at least, the switch process can take place even before VDJ rearrangement is complete (M. Wabl, unpublished).

The switch rearrangement process does not differentiate active and silent alleles. This is not surprising, since without reading the sequence, a nonproductive allele can often not be distinguished from a productive one. For instance, a silent allele may be nonproductive because VDJ rearrangement has resulted in a frameshift that will make itself apparent only upon translation in the cytoplasm. In the nucleus, such a nonproductive allele will be transcribed as well as a productive one.

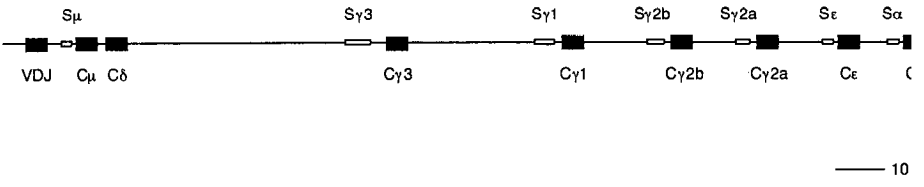

Figure 1 Organization of the immunoglobulin heavy-chain locus in a mouse B lymphocyte. VDJ, gene segment encoding the variable region; $C\mu$, $C\delta$, $C_\gamma 3$, $C_\gamma 1$, $C_\gamma 2b$, $C_\gamma 2a$, C_ε, C_α, gene segments encoding the constant region of the respective immunoglobulin heavy chains; S, switch regions located 5′ to the C gene segments.

SWITCHING VIA DNA RECOMBINATION

In the germline of vertebrates there are no functional genes encoding the polypeptide chains of immunoglobulins, only gene segments. During B cell differentiation, selected segments are joined to yield the functional heavy and light chain genes. Two types of DNA rearrangements, each of which may result in either deletions or inversions, join together gene segments that may be far away from each other in the germline (reviewed in 10, 11). The first type of rearrangement is rather precise; it is site specific and is mediated by a recombinase that recognizes known signal sequences flanking the exon segments to be joined. Exons encoding the variable (V) regions of the immunoglobulin chains and thus the antibody specificity are generated by such a process (12). The second type is rather imprecise; the recombination break points vary widely within an intron. Because the intron sequences will be deleted by RNA splicing, this imprecision does not impair the final outcome of the process. Immunoglobulin H chain class switching results from this second type of arrangement. The gene encoding the immunoglobulin H chain is reconstructed so that the gene segment encoding a given C region is replaced by another C region gene segment located further downstream in the locus. Therefore, a B cell clone can alter the effector function of the immunoglobulin produced (dictated by its C_H isotype) without affecting its specificity (dictated by its V region). After it was found that the immunoglobulin class switch results from the deletion of DNA segments (13), three different mechanisms were proposed to account for this deletion: (i) unequal recombination between homologs, (ii) unequal recombination between sister chromatids, and (iii) looping out and deletion. These are the simplest mechanisms that can account for any deletion of DNA in diploid cells. All three mechanisms may operate in B lymphocytes to achieve the switch deletion, and immunologists, as is their wont, love to point out the charms of their favorite. In the following we discuss both the positive and negative evidence for these three models.

Recombination Between Homologs

To produce a deletion, the exchange between homologous chromosomes must be unequal. If recombination occurs during or just prior to mitosis, there are four strands that can cross over. Random segregation of the four strands would result in four different types of daughter cells (Figure 2). In mice, the order at the H locus is centromere, C, V (14). Allelic exclusion dictates that only one of the two homologs encodes a functional H chain; thus, let V_A be the functionally rearranged variable region allele and V_B the silent, nonfunctional homolog. Two types of daughter cell should have two identical V segments. Cell 1 should produce both a γ and a μ chain

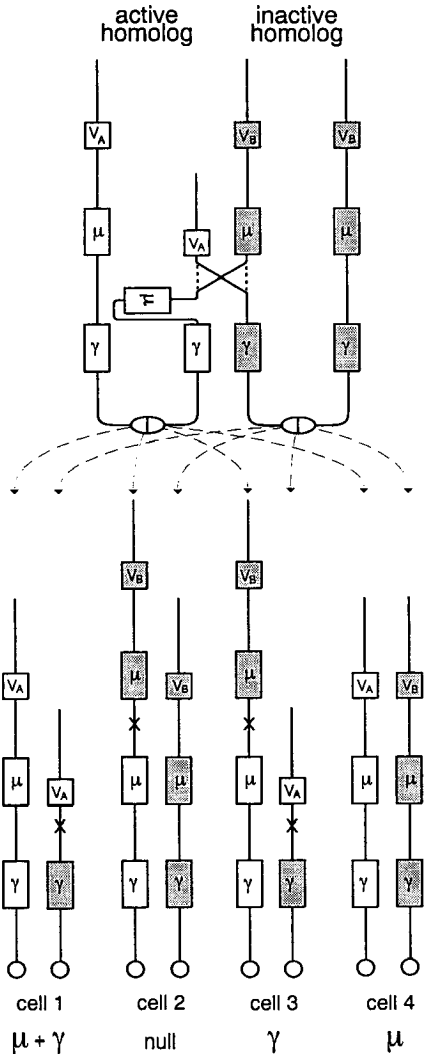

Figure 2 Unequal exchange between homologous chromosomes leading to class switch. V_A, variable region of the active homolog; V_B, variable region of the inactive homolog; [t, 11 constant gene segment; γ, γ constant gene segment representing all other constant region segments (except 8); X, recombination point; cell 1 to 4, types of daughter cells of the switching cell; the type of heavy chain a cell expresses is given underneath. The split ovals represent the centromeres, holding the two chromatids of each homolog together. In the mitosis following the switch the four chromatids segregate randomly, creating four types of daughter cells with different genomic configurations. Each daughter cell has two homologs, with one chromatid each. Shaded boxes are the gene segments originally present on the inactive homolog.

from two active alleles and should lose one Cμ segment. The other two types of daughter cell remain heterozygous with respect to V. The γ-producing cell 3 has gained on its silent homolog the Cμ that was lost by the active homolog. The μ-producing cell 4 is the only one that is inconspicuous with respect to both genetic make up and H chain production. From the above, it follows that half of the γ-producing cells should also produce μ (cell 1); the other half should contain two Cμ segments on the silent homolog (cell 3). Furthermore, among the descendants of the cell in which the switch took place, there should be half as many nonproductive cells containing three Cμ segments (cell 2) as there are γ-producing cells. In vivo, nonproducing cells should be selected against, but in vitro they might even have a growth advantage. In any event, none of these conspicuous cells have been found. They were absent in a μ-producing pre-B cell line (15), the cells of which continually switch to γ2b (9, 16). In this line there is also direct evidence that the switch rearrangement occurs in *cis* (17). If anywhere near half of the cells that produce γ, ε, or α in long-term cultures contain two active V alleles (and as a consequence also produce μ chain), and if as many cells contain three Cμ gene segments, they ought to have been noticed during the analysis of hundreds or thousands of hybridomas and myelomas that were generated in the labs around the world, by ELISA, Southern blotting, or cloning and sequencing.

Serology provides another strong argument against the possibility that recombination between homologs is the major mechanism of the switch deletion. In rabbits (18–21) and in mice (22), V and C region allotypes, i.e. serologically defined allelic products, are generally both encoded by the same homolog. But according to one report (20), as many as 8% of the molecules may have been of a recombinant type. Even if one took this figure at face value, the contribution of recombination between homologs to the switch deletion would be minor. Moreover, homologous (i.e. equal) mitotic recombination between the V and C loci would not contribute to the switch rearrangement process.

Although this mechanism of recombination is not the major source of switch deletions, it must occur at some low frequency. Indeed, illegitimate recombination between the switch regions and regions on other, non-homologous, chromosomes is known to occur sometimes, e.g. in the translocations of the oncogene *myc* to the immunoglobulin locus (reviewed in 23). However, class switching of a randomly integrated μ transgene has been reported to occur quite often (24). This might be due to trans-splicing of RNA (which is discussed below), but it can also be due to inter-chromosomal recombination (24a); it was this latter finding that rekindled an interest in this kind of mechanism generating the switch deletion. This

observation is interesting in its own right, although a μ transgene, being deprived of all *cis* sequences to which it would otherwise switch, hardly reflects the physiological situation[1]. Furthermore, the high frequency of switched cells might also be due to selection by antigen rather than to any intrinsically high rate of the process. In B cells from these mice, translocations to the H chain locus on chromosome 12 ought to be cytologically observable.

Recombination Between Sister Chromatids

Although sister chromatid exchange (SCE) is frequent in somatic cells, there is no known physiological consequence. Since it would be gratifying to assign to it a role in generating antibody diversity, the proposal that unequal SCE might be responsible for producing the switch deletion was immediately welcomed. This mechanism was proposed to explain the fact that the major intron in a γ1 producing myeloma contains a piece of the Sα region (which is the most 3′ switch region) between Sμ and Sγ1 (26). It would take at least two unequal exchanges to account for the observation, and one can imagine the participation of a number of other mechanisms, including translocation and episomal reintegration. It was also proposed that unequal SCE may have created γ2a variants from a γ2b-producing myeloma (27).

It is inherent in this model that one cannot easily disprove it. Nor is it easy to obtain direct evidence supporting it except to catch it in flagrante delicto. In an attempt to do this cytologically, we measured SCE frequencies in lipopolysaccharide-stimulated, mouse B lymphocytes (M. Wabl, unpublished). The exchanges can be directly visualized by a cytogenetic procedure. Figure 3 shows a photomicrograph of a metaphase, in this case of hamster chromosomes, with differentially stained sister chromateds. The chromated exchanges lead to a harlequin-type pattern of staining (left), which becomes more fidgety when (some) mutagens are added (right). In LPS-stimulated mouse spleen cell cultures of high cell density (2×10^6 cell/ml, or more), only IgM (together with IgD) is produced, while in cultures of lower cell density (2.5×10^5 cells/ml), the other six classes and subclasses are also produced. The SCE frequencies in both types of cultures were determined by differentially staining the sister chromatids in an attempt to correlate it with the immunoglobulin

[1] Another, clearly nonphysiological phenomenon was studied extensively by fluorescence activated cell sorter analysis of myeloma cells (25). At very low frequency, these cells, which contain more than the diploid complement of chromosomes, can switch back and forth in production of various isotypes. Recombination between homologs was implied as being the mechanism.

Figure 3 Photomicrograph of metaphase chromosomes with their sister chromatids stained differentially. Left: regular hamster chromosomes. Right: after exposure of the cell to a mutagen. Courtesy of Dr. S. Wolff, San Francisco.

class switch. In C57BL/6 mice the number of SCE in cultures of high cell density was 0.227 ± 0.016 per chromosome, as compared to 0.378 ± 0.124 at low cell density. Similar values were obtained for the Rb(4;12) mouse strain. Although the ability of lymphocytes to switch to isotypes other than μ can be correlated with increased SCE frequency, there is no dramatic increase of SCE on chromosome 12 (where the mouse heavy chain linkage group is located) over the other chromosomes: In low cell density cultures the number for chromosome 12 was 0.460 (0.409–0.525 at 95% confidence limits), as compared to 0.374, determined for over 5000 chromosomes other than chromosome 12. Chromosome 12 has medium size when compared to the other 19 chromosomes of the mouse; the slight difference in SCE frequency, as compared to the average chromosome, may be due to a slight deviation from the average size. This nominal level of SCE on chromosome 12 may be sufficient to generate a significant number of switch recombinants, yet it is clear that chromosome 12 does not stick out as being a special target for SCE. While this result by no means disproves the unequal sister chromatid exchange model for the class switch, it also does not increase enthusiasm for it.

The switch from μ to another H-chain isotype by sister chromatid exchange would have to occur at mitosis, when sister chromatids exist, and it would have to occur on the active homolog (Figure 4). The daughter cell containing the switched γ gene (cell 2) bears in its genome no mark to betray how it was generated. However, there is such a mark in its sister (cell 1), since the active homolog of cell 1 should contain two Cμ segments—what one cell loses, the other one must gain—in addition to the Cμ on the silent homolog; i.e. there are three Cμ segments in the genome.

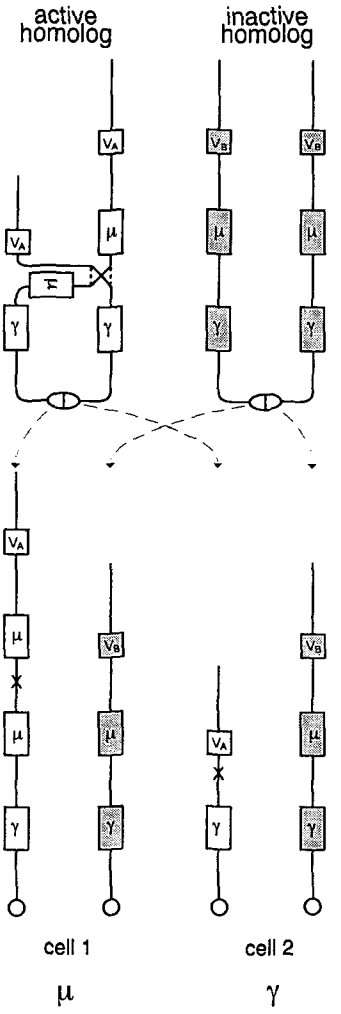

Figure 4 Unequal exchange between sister chromatids leading to class switch. For explanation of symbols, see Figure 2. Segregation during mitosis creates two types of daughter cells: cell 2 has switched to γ chain production, cell 1 still produces μ chain but contains three Cμ gene segments.

Unequal exchange on the silent homolog would also leave half of the μ^+ cells with three Cμ segments. Despite intensive searching, this configuration has not been found in the pre-B cell line that switches from μ to γ2b (15), nor in any other instance.

Looping-Out and Deletion

Looping-out is the third way to produce a deletion (Figure 5) and, as Sherlock Holmes pointed out, if all other speculations have been excluded,

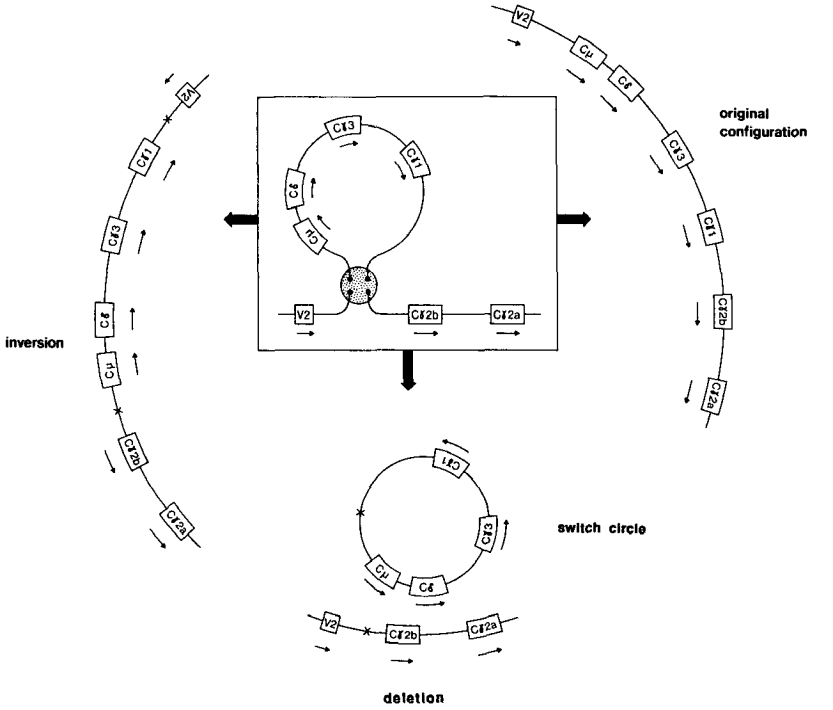

Figure 5 Looping out at the heavy-chain locus leading either to an inversion or to the original configuration or to deletion of a switch circle. X, recombination (break) point. Arrows under the gene segment define the direction of transcription. The stippled circle in the middle represents the putative enzyme complex holding the four free DNA ends together, which can be ligated in three different ways to create the chromosomal configurations shown on the sides and below. The inversion aborts any heavy chain production owing to the wrong transcriptional orientation, while the deletion of the switch circle allows expression of γ2b chain.

the remaining hypothesis must be the correct one (28). Looping out and deletion are also involved in the mechanism used to rearrange the V segments at the T cell receptor and immunoglobulin loci (29–34). After looping out and cutting, the four free DNA ends created in this way can be relegated in three different ways to produce (i) the original configuration (no switch), (ii) an inversion of the looped out sequences (loss of H-chain expression), or (iii) deletion of the looped sequences from the chromosome (switch). In the pre-B cell line we found many cells with an inversion between two switch regions (17). This was the first "positive" evidence that the switch rearrangement involves looping out. If these sequences were instead deleted, the ends of the excised 65 to 200 kb of DNA could

theoretically be ligated to form a circle; this switch circle would contain Cμ, including the 3′ part of Sμ, and all of the C regions between Cμ and the C_H to which the cell had switched. The breakpoint would be located somewhere between the 3′ part of Sμ and the 5′ part of S_H; these two switch regions, characteristic for the switch circle, would be joined in an order that is reciprocal to the configuration remaining on the chromosome.

Recently, such switch circles (Figure 6) were isolated, cloned, and partially sequenced from lipopolysaccharide-stimulated mouse spleen cells (35, 36). The alkaline lysis method, originally developed for the isolation of large Ti plasmids in bacteria and subsequently modified by Griffin et al (37) and Carroll et al (38) for Epstein-Barr virus genomes (180 kb) and large episomes in mammalian cells was efficient enough to allow isolation of rare circles (35). No contamination by chromosomal DNA was detected by Southern blot analyses or cloning into λ phages. Furthermore, from the intensities of the Southern blot bands, it was estimated that a very good yield is obtained with this simple and fast method (0.25 to 0.5 circle per switching event, if one assumes that the circles do not replicate). These results were quickly reproduced by Sakano et al (39).

As none of the genomic configurations predicted by recombination between homologs or sister chromatids could be demonstrated, while both products of looping-out—inversions and circular DNA—could be isolated, it would seem that the chapter on the question of which mechanism produces the switch deletion can be closed.

DO THE SWITCH CIRCLES REPLICATE? The origins of replication on animal chromosomes are estimated to be 50 to 330 kb apart (40), so it would not be surprising if there were an origin somewhere within the approximately 200 kb of DNA containing the segments encoding the various loci of the immunoglobulin H chain C regions. In a pre-B cell line in which cells had switched to γ2b on both alleles and, thereby, had deleted Cμ from the chromosome, no Cμ sequences were found by polymerase chain reaction (U. von Schwedler, M. Wabl, unpublished observation); thus, the circles excised during a switch to γ2b and containing Cμ-Cδ-Cγ3-Cγ1 do not seem to replicate, at least not at the same rate as do the chromosomes. In switch circles containing the Cγ2b, Cγ2a and/or Cγ, exons would be much larger and might well contain an origin of replication that also functions extrachromosomally. In non-B cells, the immunoglobulin H locus functions as a single replicon with its origin downstream of Cα (41); however, additional origins of replication may be activated in B cells.

Although the intervening sequences between the C_H exons have been sequenced, one does not know whether any additional genes are located in this region. For example, an open reading frame has been found between

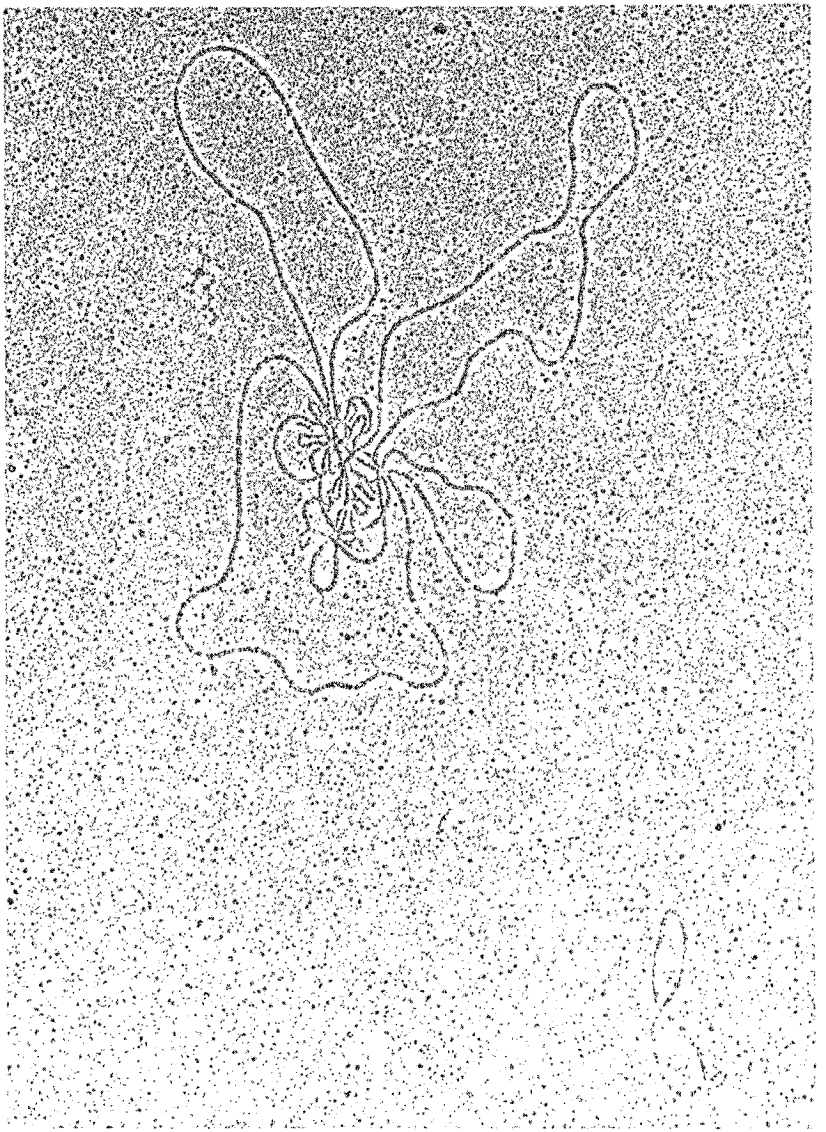

Figure 6 Electron micrograph of an isolated circular DNA molecules. The DNA molecule in the center is larger than 100 kb. The figure-eight shaped small molecule at the lower right represents a pBR 322 molecule and serves as a size marker.

the Cμ and Cδ (42). If the maintenance of expression of these putative genes were to be crucial for the switched cell, there would be a strong selection for the continuous presence of some switch circles.

WHAT DECIDES BETWEEN DELETIONS AND INVERSIONS? As explained above, deletions and inversions are two different ways to resolve the looping out of DNA for the recombination process. In some hybridomas the active allele has a switch deletion, and the silent allele has a switch inversion (43). In the Abelson virus-transformed pre-B cell line, where this phenomenon was studied and where there is no selection, there are almost as many inversions as there are deletions (17). The reason for this is unknown, and it is only paraphrasing the experimental observation to say that the switch recombinase is a sloppy enzyme.

Homologous vs Site Specific Recombination

It has been argued that the repetitive sequences of the S regions facilitate homologous recombination (44) or focus the putative switch recombinase (45). Thus, the switch regions may help to juxtapose DNA by virtue of their homologous sequences, or they may be recognized by and bind to a component of the switch recombinase. The discovery of switch inversions makes nonhomologous, site-specific recombination more likely, because homology is probably not maintained in the other reading direction. But switch regions may contain inversions in their genomic configuration. And, of course, it is very difficult to exclude homologous recombination, if one sets the degree of homology low. General eukaryotic recombinases can join DNA sequences with as little as 13 base pairs of continuous identical sequences, or sets of three smaller patches separated by deletions and/or looping outs (46, 47). Still, recombinational breakpoints do not appear to occur preferentially in switch regions of maximum homology (48). How many base pairs of homology would be needed for switching? At a minimum, one, in which case the controversy resolves into almost a semantic one.

The sequences of the mouse S regions consist of repetitive elements that vary in length from 10 to 80 base pairs. S regions span anywhere from 1 kb (Sε) to 10 kb (Sγ1). Each switch region has its own characteristic short and long repeats, yet all share homology with one another. As compared to Sμ, the degree of homology is highest with Sε, and then decreasing in the following order: Sε > Sα > Sγ3 > Sγ1 > Sγ2b > Sγ2a > (49, 50). It is found that recombination with the switch region most homologous to Sμ is the least frequent: Sε. This would speak against homologous recombination; but Sε is also the shortest, and cellular selection might bias the frequencies. One can also argue that first the locus has to be "open," and only then would homology become important.

Transcription as a Requirement

In both human and mouse, switching is preceded by transcription of the unrearranged C gene segment to which the cell will be switching (51–65). Since they appear to be not translated, these germline transcripts are also called "sterile transcripts"; however, an open reading frame has been found in the germline μ (66), γ2b (54), and α transcript (67). The transcripts are initiated at multiple sites, about 2 kb 5′ to the S region, and proceed through the S region and C segment (54, 56–58, 60, 61, 68, 69); thus they lack the VDJ segment. The question is, are these transcripts a prerequisite for the switch recombination or are they fellow travelers of it? An argument for their being required is the strong correlation between their induction by a particular interleukin and the subsequent specific class switch directed by that interleukin; e.g. IL–4, which directs spleen cells to switch predominantly to IgG_1 or IgE, also induces germline γ1 and ε transcripts (55–57, 64, 65, 68–72). However, there are also examples where this correlation does not hold (65).

In yeast it has been shown that transcription by DNA polymerase I or II can stimulate recombination more than 15 fold (73–75). Recently, Leung & Maizels (76) have directly tested the effect of transcription on class switching in mouse cells. The presence of an upstream transcriptional control region seems to increase the recombination frequency of an extrachromosomal substrate at least 10 fold.

SWITCH RECOMBINASE

Now that the over-all mechanism of switching has been solved, an important—if not the most important—immunological question remains: How is the class switch directed? Because the difference between switching to IgG and switching to IgE is the difference between immunity and allergy, this question also has great clinical relevance. An authoritative review on the role of interleukins, i. e. on the role of extracellular factors was provided by Finkelman et al (77). A detailed answer to the question will also require the identification of the intracellular molecular components responsible for the immunoglobulin H chain class switch. We assume that the switch rearrangement process, like other biochemical reactions within the cell, is facilitated by an enzyme(s). This putative enzyme has been named *switch recombinase*. Nothing is known about its components. The switch recombinase must perform several of the same functions as the VDJ recombinase, which generates the VDJ exon from three different germline gene segments. These functions include binding to DNA, cutting it, and ligating it. The two enzymes obviously differ in their DNA recognition sequences. For the

VDJ-recombinase, these are the heptamer-nonamer recombination signal sequences, which flank the gene segments (reviewed in 12). For the switch recombinase the recognition sequences are not known, but the simplest assumption is that they are part of the switch region. Since the cutting and ligating activities are common to both enzymes, they may even be executed by the same proteins, but the binding component must be different. Although no breakthrough similar to the one for the VDJ-recombinase—the characterization of the *scid* mutation and the cloning of RAG–1 and RAG–2—has been made, the regions where most of the recombination break points are located (the switch regions) have been identified long time ago (10). Some sequence requirements for the recombination process have been established (76, 78), and some binding proteins thought to be involved in the switch rearrangement process have been described (see below).

Binding Proteins

A popular hypothesis on directed class switching proposes that specificity is provided by the so-called "sterile transcripts" originating 5′ to the repetitive elements of the S regions (see above). This hypothesis is based on the observation that the VDJ recombinase is the same for B and T cells, yet only the genes for the appropriate antigen receptors are, in general, rearranged. It was argued that either the immunoglobulin locus or the T cell antigen receptor locus is "accessible" in a given cell, but not both. Logically, there is no difference between the assumption of a core recombinase plus several different factors guiding the recombinase to a given locus and the proposal for different recombinases working at the various loci. For the accessibility hypothesis to be more than a tautology, it must specify what makes the loci accessible. If specific transcription provides the specificity of rearrangement, then the transcription factors may be considered part of the putative switch recombinase complex. The same may be said about the factors of a putative (de)methylation system that would be responsible for the accessibility of recombination sites (79–81). Although accessibility of this region may make it a (specific) target for recombination, there must be, in addition, specific factors that direct the recombination to two different switch regions, but not to other—also transcribed—sequences that are very similar to each other. These factors need not be specific for a particular switch region, but they ought to be specific for a generic switch region.

It is unclear whether a given recombinase, or its binding component, recognizes all switch regions or only a subset thereof. It is also not clear whether or not the essential elements of the switch regions reside in the

portion with the repetitive sequences; but the most straightforward assumption is that the switch region itself contains the binding site. It was reported that the γ1 switch region contains repetitive octamer-like sequences (82) and that the sequences flanking those repeats are important for protein binding. Several other laboratories have described similar factors (83–86). Some of them are LPS-inducible and bind outside the (repetitive) switch regions.

Such binding factors were commonly identified with the gel mobility shift technique. In conjunction with protection and methylation interference assays, this technique was often used to identify DNA sequences that are essential for a specific protein-DNA interaction. However, the gel shift assay is not sensitive enough to exclude with certainty small but functional levels of a protein. Therefore, the moment of truth, in regard to tissue specificity, for binding factors identified by gel shift assays comes when they are cloned and when different tissues are assayed for mRNA encoding the factors. Only then does it become clear whether or not their expression is restricted to the appropriate B cell differentiation stage(s). At the time this review was written, no such cloning has been reported.

A more tedious task is to demonstrate that such factors directly contribute to the switch rearrangement process, i.e. they are part of the switch recombinase complex. But once a binding protein is cloned, there are obvious ways to assess whether it is at all a necessary component for class switching. For example, knocking out the gene encoding the binding protein in the cell line that continually undergoes class switching would provide an answer. Or the cDNA can be cloned into a mammalian expression vector, and then stably or transiently transfected into a cell line that does not switch its immunoglobulin isotype, e.g. the 3T3 fibroblast line, a plasmacytoma, a hybridoma, and a B lymphoma. It then can be tested whether or not the cells switch, or whether a cell line switching to one isotype now (exclusively) switches (also) to the isotype targeted by the binding protein.

Cloning by Function

The stunning (and, so far, only) success in cloning (parts of) the VDJ recombinase came from cloning by function (87, 88). Although not many researchers would have bet on this particular approach, it was facilitated by the extensive work that had been done on recombination substrates with their defined heptamer-nonamer signal sequences. Difficulties with designing an appropriate switch substrate stem from the fact that there is no agreement on the signal sequences for the switch recombination.

Therefore, large fragments may have to be used. Unfortunately, such substrates may be difficult to generate due to the highly repetitive nature of the switch regions; classic cloning of the entire region often results in deletion of part of the sequences, and cloning of smaller segments is problematic since there is a lack of suitable restriction sites. However, a retroviral vector containing Sμ and Sγ2b was used to monitor switching in the Abelson virus–transformed pre-B cell line 18–81 (78). The switch frequencies reported with this substrate were moderate, but in another vector with switch regions lifted off from the germline there were no problems with "spontaneous" deletions, and switch frequencies were high (76). The experimental approaches described above focus on different aspects and functions of the putative switch recombinase. The isolation and characterization of a specific switch region binding protein must be complemented by a functional assay that demonstrates that the protein is part of the recombinase. And the isolation of factors that promote recombination in a switch substrate must be followed by an analysis that differentiates between transcription factors and enzymes. Success with a set of experiments designed to isolate the one factor may make inroads into the hunt for the other component. For example, the first specific binding protein can be used to pull out further proteins, which are bound to the first one and which are also components of the recombinase.

SWITCHING WITHOUT DNA RECOMBINATION

Alternative Splicing of Long Nuclear RNA

As soon as introns were discovered, it was suggested that immunoglobulin class switching is due to alternative splicing of long nuclear RNA (89); and for hybridomas it was almost immediately ruled out that RNA splicing is the mechanism of producing mRNA encoding an isotype other than μ (89a). However, alternative splicing is thought to be the mechanism by which small resting B lymphocytes produce both IgM and IgD (90, 91), although the predicted 28 kb RNA has yet to be isolated.

Alternative splicing is also an attractive idea to explain the existence of the lymphocytes producing two isotypes that are generated after stimulation with bacterial lipopolysaccharide in vitro. After 6 hr of stimulation of mouse spleen cell cultures at cell concentrations lower than 10^6 per ml (6), many B lymphoblasts begin to produce IgM as well as immunoglobulin of another isotype. But are the mRNAs encoding each isotype still being produced in these cells, or is the μ chain translated from still undegraded mRNA that was transcribed *before* an isotype switch? Attempts in several laboratories to rescue the double-producing lymphoblasts by fusion to cells of a similar differentiation stage, e.g. to a hamster B cell lymphoma

synthesizing IgG, have failed; each hybrid cell produced either IgM or another isotype, but not both. However, one can argue that the fusion event forces the previously double-producing cell to produce only one isotype. In any event, proof for the alternative splicing of a long transcript (approximately 200 kb for mouse α chain) would clearly have to include proof for the existence of such a long transcript. Although it was reported that sorted spleen cells producing both IgM and IgG1 contain RNA segments transcribed from intervening sequences between the segments encoding the various H chain C regions (92), there seem to be no other papers following up on this line of experimentation. We note that switching in LPS-stimulated spleen cells is accompanied by the classical switch deletion of DNA (93).

Unless continuous production of two isotypes (other than IgM and IgD) is documented (e.g. by sorting, stripping, and allowing resynthesis), the absence of DNA rearrangement is only a weak argument for alternative splicing of a long transcript. For instance, most, perhaps all, resting B cells that display both IgM and IgE on the surface do so because IgE is absorbed onto the membrane (94). And a population of sorted cells will contain many cells with unique histories of recombination, which will preclude the detection of a specific restriction fragment length polymorphism indicative of switch recombination. Thus the argument must rely on not seeing any less than exactly two unswitched alleles. In cell lines that produce an isotype other than μ, the absence of DNA rearrangement would seem to be a better argument for the existence of alternative splicing. However, the literature is replete with examples of failure to find DNA rearrangement because of experimental errors.

Because there exist genes that span well over 100 kb and that are, presumably, transcribed as a single continuous RNA molecule, there is no reason a priori to dismiss the notion of a transcript long enough to allow alternative splicing as a mechanism for the immunoglobulin class switch. However, there still seems to be no convincing demonstration that such a mechanism can account for the immunoglobulin class switch in vitro or in vivo, even in special cases.

Trans-splicing

Trans-splicing as the mechanism for some switch events is a novel and exciting suggestion. A variant of the BCL1 cell line, which produces both IgM and IgD, has switched to IgG1, while maintaining IgM production (95). A detailed Southern blot analysis of the entire C region locus failed to find a switch rearrangement (95). However, the analysis is complicated by the fact that BCL1 has lost the silent homolog of chromosome 12 and duplicated the active homolog, i.e. there are two alleles containing

identical, functionally rearranged VDJ exon linked to the segments encoding the H chain C regions. While there is no rearrangement on the one allele, which then presumably encodes the μ chain, there is a large deletion on the duplicated allele just 3′ of the VDJ exon. Since no Cγ1 segment could be found to be linked to this VDJ exon, it was concluded that this allele does not encode the γ1 chain. It would be interesting to switch the BCL1 cell line repeatedly in independent switching events. If the deletion on the one-"inactive"-allele is strictly correlated with the switching, this would indicate the possibility that it constitutes indeed a switch deletion giving rise to γ1 expression. Conversely, if μ and γ1 are encoded on one unrearranged allele, they should segregate together when chromosomes are lost.

In a follow-up study, Nolan-Willard et al (96) reported that the IgG1-producing BCL1 subclone synthesizes pre-mRNA that contains both μ and γ1 sequences. They thus propose that the simultaneous μ and γ1 RNA synthesis is accomplished by discontinuous transcription followed by either trans-splicing or ligation of μ pre-mRNA to sterile γ1 transcripts. While this report shows unassailable sandwich hybridization blots, proof for the presence of the putative pre-mRNA would require a cDNA sequence.

In mice with a human μ transgene lacking other C gene segments, stimulated spleen cells were reported to synthesize both human μ and mouse γ chains (97). A problem of allelic inclusion was ruled out by the authors. Because the transgene was not integrated on chromosome 12, the authors also excluded an intrachromosomal deletion as an explanation. Rearrangement of neither the transgene nor the endogenous H chain locus was found in the sorted μ plus γ-producing blast cells. Of 12 lymphomas screened, one did not produce μ, but apparently contained an mRNA encoding the human transgenic VDJ linked to mouse Cγ2a segment. This led the authors to conclude that trans-splicing is responsible for the class-switch in this particular case. The presence of this trans-spliced message was demonstrated by RNase protection; however, the actual sequence of the VDJ-γ1 junction was derived from a PCR amplified fragment, rather than from a cDNA clone. PCR is prone to many artifacts, including template switching. As stated above, isolation of cDNA encoding a trans-spliced mRNA would make one feel much more comfortable with the unorthodox conclusion. There should be no difficulty in doing that in a lymphoma line.

We have to await confirmation of these intriguing findings before such a mechanism is awarded a suitable place in the panoptican of events that can happen. However, in its simplest form, such a mechanism cannot account for the majority of switch events, at least in rabbits and in mice, where there are convenient serological markers for both V region and C

region allotypes (see above). In heterozygotes, over 90% of serum IgG does not show scrambled markers; i. e. when the V region of an immunoglobulin H chain is of haplotype *a*, so is the C region of that molecule, and when the V region is of haplotype *b*, so is the C region. Were trans-splicing responsible for the class switch, we would expect a V region of haplotype a to be associated with a C region of haplotype *b* in about 50% of the H chain molecules.

CONCLUDING REMARKS

The ideas that are stronger than experiments are called paradigms (98). From this definition it follows that one cannot disprove a paradigm with experiments, one can only try to replace it with another paradigm. It is interesting to note that paradigm articulation seems to proceed differently in the similar problems of VDJ rearrangement and switch rearrangement.

Although unequal sister chromatid exchange had been suggested as the mechanism for rearrangement of Vκ to Jκ, this mechanism was dropped in favor of the looping out and deletion mechanism when the circular excision products stemming from the T cell antigen receptor V segment rearrangements were isolated. For the generation of the chicken V exons, the demonstration that signal joints could be found in Bursa cells by PCR was accepted as a proof for the looping out and deletion mechanism, even though the very same joints are predicted by the other two competing models.

However, for the heavy chain class switch the models seem to coexist happily together. To the best of our knowledge, no one is making a V_H transgenic mouse to see whether the exogenous V_H can recombine with an endogenous D and thus collect evidence for recombination between homologs or trans-splicing. Moreover, a protein that binds to a switch region but that is found in all cells, and even in *Drosophila*, would probably not create the same excitement as the ubiquitous heptamer binding protein implicated in the VDJ recombination process. The discovery of the switch deletion, reported 15 years ago by Honjo in a landmark paper (13), was easily accepted because it was preceded by analogous findings for the generation of the V exons. Indeed, except for some variations on the theme, it can be said that until now research on switch rearrangement has followed in the wake of research on VDJ rearrangement. We predict that there will be a role reversal in their application to clinical immunology. For example, when the putative intracellular factors guiding the switch recombinase specifically to S_ε are cloned, they can be used to screen for small molecules that bind to them, and thus they may be pharmacologically active in

preventing the switch to IgE. Such small molecules would act as universal anti-allergens.

ACKNOWLEDGMENTS

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THE MOLECULAR BASIS OF ALLORECOGNITION

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Abstract

Until recently it was generally assumed that the focus of T cell receptor recognition of allogeneic MHC molecules was the polymorphic regions on the molecule that differed between responder and stimulator. It is now clear that all T cell recognition, including self-tolerance and allorecognition, involves both the MHC molecule and its associated peptide ligand. Polymorphic residues located within the peptide binding groove of the MHC and inaccessible to the T cell receptor can profoundly affect selection and recognition of bound peptides. These peptide differences between histoincompatible individuals greatly amplify the antigenic impact of MHC polymorphism and result in the high frequency of alloreactive cells. Evidence for the role of peptides in allorecognition is reviewed.

INTRODUCTION

The basis for recognition of alloantigen is one of the most historically significant issues in immunology (1, 1a). Indeed, the identification of the targets of allograft rejection led to the definition of MHC gene products. The vigorous T cell response observed during graft rejection, and its in vitro correlate, mixed lymphocyte culture, has also been one of the most perplexing issues in immunology. In contrast to the low frequency of T cells that recognize conventional antigens, as many as 1–10% of T cells respond to an alloantigen (2–8). While this finding provides a rationale for

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the vigor of an alloresponse, the molecular basis of this extraordinarily high frequency of recognition of alloantigens has eluded immunologists. Over the years, a number of different theories have been proposed to explain this high frequency of alloreactive T cells. Due to recent progress in our understanding of MHC structure and function, and the composition of the T cell receptor repertoire, much of the mystery has been solved. It may, therefore, be an appropriate time to revisit the most prominent of these theories and place them in the framework of current knowledge.

THEORIES TO EXPLAIN ALLORECOGNITION

Germline Encoded Allorecognition

One of the earliest attempts to explain the basis for the high frequency of alloreactive cells was described by Jerne (9, 10). This theory proposed that the repertoire of lymphocyte receptors was evolutionarily preselected to include germline genes that had specificity for the MHC molecules of the species (9, 10). According to Jerne's theory, for each MHC molecule there evolved a complimentary receptor molecule. If there were 100 different MHC molecules in the species, then correspondingly there would be 100 different germline receptor genes. This readily explained why 1% of lymphocytes respond to each MHC molecule. Jerne further proposed that the receptor repertoire used in recognition of foreign antigen was derived by somatic mutation of the receptor specific for self MHC molecules. This would both avoid autoreactivity and provide the diverse repertoire required for immune surveillance.

A prediction of this model was that the receptor repertoire specific for each alloantigen would consist of as few as one type of germline encoded receptor. In essence there would be two types of clones within the repertoire. One would consist of very large clones that expressed receptors encoded by germline genes (allospecific clones), whereas the other would be small clones of somatically mutated receptors that were devoted to recognition of the rest of the antigenic universe. This would simultaneously explain the high frequency of alloreactive CTL and the low frequency of antigen specific CTL. A second prediction of this theory was that the repertoire used in recognition of antigen was separate from the allospecific repertoire.

The MHC as an Interaction Antigen

Matzinger & Bevan (11) also developed a hypothesis that attempted to explain the high frequency of alloreactive cells. Their hypothesis was based on the observation that CTL could be obtained between MHC identical strains that differed from each other with respect to expression of minor

antigens (12). At the time, these antigens were believed to represent polymorphic cell surface proteins. According to this model, the MHC was an interaction molecule that could complex with essentially all other cell surface proteins, and this complex of MHC plus X (where X represents a cell surface protein) was presented for recognition by the T cell receptor on CTL. In the case of recognition of foreign antigens interacting with self-MHC molecules, the MHC portion of the complex was self, and a polymorphic minor antigen or viral antigen was foreign. However, T cell receptor recognition could also be obtained if X were self and the MHC component of the complex was foreign. Considering that hundreds of different proteins may be expressed by the cell and available for interaction with the MHC, the high frequency of alloreactivity was a consequence of the diversity of antigenic complexes available for recognition. Another important assumption of this hypothesis was that no restriction was placed on the extent of diversity permitted to the T cell receptor repertoire, because there had to be many different receptors available in order to respond to each unique interaction complex. In contrast to the theory of Jerne, the Matzinger-Bevan hypothesis permitted a high degree of receptor diversity within the alloreactive population as well as no conceptual restrictions on overlap between the allospecific and self-MHC-restricted antigen specific repertoires.

This theory allowed for a testable prediction. Although it was considered likely that the majority of alloreactive T cells were specific for cellular antigens that were nonpolymorphic, the fact that minor specific CTL existed implied that at least some interaction proteins were polymorphic. Allo CTL clones specific for these antigens would not be expected to recognize targets that expressed the appropriate allo-MHC yet lacked the appropriate allelic form of the minor antigen. One reported attempt to identify allospecific CTL clones with such specificity proved unsuccessful (13).

Evidence That the T Cell Receptor Repertoire Specific for Alloantigen Is Diverse

The issue of the number of different types of epitopes recognized on a single alloantigen was of great significance in understanding the magnitude of an alloresponse, as well as in distinguishing between the theories described above. If many different epitopes were presented by a single allo-MHC, then it was less likely something intrinsically unusual existed about the allospecific repertoire as compared with the repertoire specific for conventional antigens. Initial attempts to assess diversity within the allospecific repertoire capitalized on the availability of a series of mutant mice that differed from wild type (C57BL/6) in that they carried mutations in

their K^b molecule (14). By studying the specificity of CTL induced in response to wild type or mutant K^b molecules, it became clear that most mutants had both gained and lost some epitopes relative to wild type (14, 15). Using the newly established technology of T cell cloning, it became possible to study in detail the specificity of individual K^b specific clones using a panel of K^b mutant targets to identify and discriminate clonal specificities (16). With this approach the diversity of the receptor repertoire against a single class I molecule could be assessed. The extent of receptor diversity among K^b-specific CTL clones was extensive, as based on recognition of many different epitopes on this single alloantigen. Furthermore, even when the difference between stimulator and responder was limited to a small number of amino acids, as in the case of responses between C57BL/6 and mutant strains, many different receptor specificities could readily be discriminated by this method (17, 18). Superimposed on this extensive diversity, several specificities were observed that recurred with high frequency, suggesting that the level of representation of the various receptors could vary greatly (16, 19). Nevertheless, results such as these argued strongly against the idea that a small number of germline genes was responsible for recognition of each alloantigen.

Current technology has allowed assessment of repertoire diversity at the level of expression of different T cell receptor genes, confirming that a large number of receptor genes are expressed in response to a single alloantigen (20–22). Of interest, several reports observe that one or several $V\beta$ recur with high frequency within a particular alloresponse. However, many receptors that share V_β probably recognize a variety of different epitopes, as often no conservation of the hypervariable regions occurs among these receptors (21, 22).

Alloreactive T Cells Are Part of the Antigen Specific Repertoire

Another important observation concerning the allospecific repertoire resulted from the body of evidence demonstrating that alloreactive CTL were part of the repertoire devoted to recognition of antigen in the context of self-MHC. Once it was possible to obtain and propagate antigen-specific T cell clones, those clones restricted by self-MHC were frequently observed also to display alloreactivity (23–25). Molecular analysis further demonstrated that the same portion of the TCR involved in recognition of antigen plus MHC was involved in recognition of alloantigen (23). Thus, the antigen specific and allospecific repertoires are one and the same.

The ability of individuals to respond to foreign antigens presented by self-MHC molecules is attributable to the fact that the T cell receptor repertoire undergoes positive selection for cells that can recognize self-MHC with low affinity (26–29). What then is the origin of the repertoire

that recognizes allo-MHC molecules? Presumably this represents cross-reactivity by cells that were originally selected on the basis of recognition of self with low affinity.

SOME OTHER THEORIES

Recognition of Conformational Alteration of MHC Molecules

In the course of analysis of a number of CTL clones of B6 origin that were specific for a particular K^b mutant, bm11, many of these clones recognized a second K^b mutant that was altered at a different amino acid position on the K^b molecule (18). For example, 24% of bm11-specific CTL clones obtained from B6 responders were able to recognize bm1 targets. These two mutants carried amino acid substitutions in different domains of the molecule (14). Thus, mutational alterations occurring at different locations in the molecule apparently could result in formation of similar epitopes. For both bm11 and bm1 to be recognized, the receptor must have focused on a portion of the K^b molecule shared by these mutants. Wherever that portion was, it was by definition identical in sequence to wild type and therefore existed in both the responder (wild-type) and stimulator (mutant). Considering that these clones were not autoreactive, the basis for the antigenicity of a self-epitope was likely to be availability to the receptor. This could be explained by a conformational difference between the mutant and wild type molecules that allowed a portion of the MHC to be exposed in the mutants that was not available for T cell recognition in the wild type. Based on these considerations, it was proposed that mutation at several different places on the K^b molecule could result in formation of equivalent conformational epitopes (18).

The implications of this theory extended beyond allorecognition. If a mutation in class I could cause conformational changes recognizable by T cells, then antigen interacting with class I could also cause a conformational alteration resulting in recognition by some T cell receptors. Thus the recognition of a complex comprised of self-MHC plus antigen became reduced to recognition of a conformational change in the MHC per se.

Affinity Model To Explain Allorecognition

The preceding two theories of allorecognition were based on the assumption that the epitopes recognized by alloreactive cells are equivalent in antigenicity to those recognized during conventional T cell responses and that the difference in the magnitude of these responses was attributable to the large number of epitopes present on an alloantigen. However, an alternative view considered the antigenic strength of alloantigen "as a

consequence of the density of antigenic molecules on the target cell," a notion stated and dismissed early on by Simonsen (30), yet later reconsidered by Bevan (31). Bevan proposed that unlike conventional foreign antigens, which must compete with the multitude of cellular epitopes to interact with MHC for presentation to T cells, and, therefore, would be found associated with relatively few class I molecules, the foreign sequences on an allo-MHC were present on all class I molecules and therefore were far more abundant. This high concentration of antigen could permit stimulation of many T cells of relatively low affinity. Thus the difference between alloreactive T cells and those responsive to conventional antigen was related to the range of affinity of the receptors that were stimulated. Receptors reactive to conventional antigens had to have a very high affinity, a criteria met by relatively few T cells, whereas in contrast, alloreactive T cells would require a much lower affinity for activation, a criteria met by a higher proportion of the receptor repertoire.

THE STRUCTURE OF THE MHC MOLECULE

As some of these theories suggest, a key issue in understanding the molecular basis for allorecognition is the origin of the vast diversity of antigenic epitopes presented by a single MHC molecule, particularly in the case of the K^b mutants where there are only one or a few amino acids that differ between responder and stimulator MHC . When the structure of the HLA-A2 molecule was solved by X-ray crystallography, an important key to this puzzle was revealed—that the polymorphic residues of the molecule participated in the formation of an antigen binding groove that contained a heterogeneous population of peptides (32–34). Thus, the product of a single MHC gene was not a homogeneous population of identical structures; rather, these molecules contained an assortment of 100s to 1000s of different peptides in their peptide binding grooves (35–37). As the rules governing processing and presentation of these peptides unfolded, it became clear that the reason MHC polymorphism was focused on residues that lined the peptide binding groove was to diversify the sequence of peptides able to bind each of the various MHC molecules. In order to bind a particular MHC molecule, the sequence of the peptide had to conform to a certain motif (38). Therefore, an individual could increase the number of peptides presented for T cell recognition by expressing more than one MHC molecule, and the species could further increase the potential for immune protection through MHC polymorphism.

Interestingly, the K^b mutants had their mutations in the peptide binding groove of the molecule and thus could change the constellation of peptides that bound K^b (78). Involvement of peptide as part of the epitope recog-

nized by the T cell receptor could explain the diversity of antigenic determinants recognized by alloreactive CTL

The existence of the peptide binding groove provided an explanation for the strength of an allogeneic response that was reminiscent of the model originally proposed by Matzinger and Bevan. The high frequency of alloreactive T cells could indeed reflect the diversity of "interaction structures" comprised of the MHC molecule and each of a number of different cellular peptides. Of interest, an important and unanticipated new level of diversity in this early model was the fact that the actual peptides that could interact with the MHC molecule potentially differed for each MHC molecule. Thus, a judiciously selected single amino acid change in the peptide binding groove of the MHC could, in theory, lead to great diversity when the result of that change was binding by a different set of peptides.

The concept that a variety of different peptides could bind a single alloantigen also provided a framework for a new interpretation of the earlier model proposing recognition of conformational determinants on the MHC. Specifically, whereas it was originally proposed amino acid differences between class I mutants could lead to conformational differences detectable by the TCR, as recently proposed by Bluestone (39), binding by different peptides may also result in conformational differences in class I detectable as alloepitopes. Of interest, the fact the K^b mutants affect peptide binding provides an alternative explanation for the observation that unrelated mutants can be recognized by the same CTL clone. For example, the K^b molecules in both bm1 and bm11 may bind a peptide that cannot bind to the wild type molecule.

EVIDENCE FOR THE ROLE OF ENDOGENOUS PEPTIDE IN ALLORECOGNITION

The first experiments to suggest that peptides bound by the MHC were involved in allorecognition focused on class II MHC molecules. Incubation of class II–presenting cells with exogenous antigen was observed to reduce the ability of alloreactive T cells to recognize class II MHC molecules. This result would be expected if high concentrations of exogenous antigen inhibited presentation of a different peptide required for allorecognition (40–41). One study observed that most allospecific T cells were affected by high concentrations of an influenza peptide that specifically bound the class II molecule normally recognized by these T cells (40). Demotz and coworkers (42) reported an elegant demonstration that class II was necessary but not sufficient to stimulate allospecific T cells. An affinity chromatography procedure specifically purified complexes of class II ($I\text{-}E^d$)

occupied by a hen egg lysozyme peptide. These complexes were highly efficient at stimulating lysozyme specific T cells, but inactive in stimulating alloreactive T cells.

Another kind of observation that suggested peptide involvement in allorecognition was tissue specificity in recognition of a murine class II molecule. In both the murine and human systems, some cells expressing the appropriate class II were unable to stimulate T cell clones (43, 44). These results suggested the specific class II molecule was necessary but not sufficient to account for allorecognition, a fact which could be explained by tissue differences in expression of proteins that contribute peptides for presentation with class II. For example if a T cell clone was specific for class II that contained a peptide derived from a protein uniquely expressed in B cells, than it could not respond to macrophage that express the appropriate class II molecule but not the requisite peptide.

Direct evidence for peptide involvement in allorecognition was first obtained for class I (45). A panel of K^b specific CTL clones was tested for the ability to lyse a human cell line transfected with K^b (Jurkat K^b). This test sought evidence for peptide dependence by searching for a CTL clone that recognized a murine peptide not expressed in human cells. Species specificity in allorecognition had been observed previously (46–48). Although the majority of K^b specific clones were able to recognize K^b expressed by the human target, several could not. To determine if this lack of recognition was due to the lack of presentation of a specific cellular peptide, cytoplasmic proteins were prepared from a murine cell line, cleaved with cyanogen bromide to produce peptides, and these peptides were then used in an attempt to sensitize Jurkat K^b targets for lysis by these same CTL clones. Such peptides were indeed able to cause lysis of the human cell line.

These data demonstrated that at least some T cells were peptide dependent in their recognition of alloantigen. However, the generality of peptide dependent allorecognition could not be assessed from these studies. One indication that other clones were also peptide dependent came from analysis of a variety of different murine targets that expressed K^b. As previously observed in the recognition of allogeneic class II, expression of the appropriate class I MHC molecule was necessary but not sufficient for recognition by a number of K^b specific clones (49).

A major advance in this area was the identification of mutant cell lines such as T2 and RMA-S that were defective in processing and transporting of endogenous peptides (50–53). In particular, a derivative of one of these lines that was transfected with the K^b gene (T2-K^b) expressed relatively normal levels of K^b on its surface; however, these molecules were devoid of peptide. When this cell line was used as a target for recognition by K^b

specific CTL clones, most of these clones could not recognize the empty K^b molecules expressed by T2-K^b (54). One clone (bm8.10) was able to recognize T2-K^b, but the level of lysis varied greatly from experiment to experiment and could be improved 10–100 fold by pulsing T2-K^b targets with CNBR-cleaved cytoplasmic proteins. Thus even clones that appeared to recognize T2-K^b might be peptide dependent in their recognition of K^b. Their ability to lyse T2-K^b in the absence of peptide could be due either to a low affinity for empty class I or perhaps to the ability of the T2 cells to present some peptides despite their defect in processing.

The availability of cell lines such as T2 and RMA-S also provided an excellent opportunity to identify peptides required for allorecognition (54, 55). Many of the K^b specific CTL clones unable to recognize these mutants could do so after these cell lines were exposed to cellular peptides that had been obtained either through CNBR cleavage of cytoplasmic proteins or through acid extraction of processed peptides from whole cell lysates or purified K^b molecules. Indeed, combining this approach with HPLC fractionation of cellular peptides revealed that each K^b specific CTL clone recognized a different cellularly derived peptide or set of peptides. Figure 1 represents the results of such an experiment in which peptides extracted from K^b molecules purified from EL-4 tumor cells were fractionated by HPLC, and each fraction was used to pulse T2-K^b targets. Several CTL clones such as clone 13, M6, and 30 recognize a single peptide peak whereas others such as 72 and bm8#28 recognize peptides that appear in more than one peak or elute over a broad range of salt concentration.

The significance of such multiple peaks or broad peaks is not yet clear. This could indicate that more than one peptide sequence can be recognized by a single allospecific clone. Some clones may require peptide occupancy of MHC for their recognition yet may exhibit degeneracy in peptide specificity such that any one of a number of peptides will serve equally well to restore recognition (discussed further below). Alternatively, several peptides of slightly different length may contain the requisite sequence. As an example, in a recent report by Udaka and co-workers (56) one CTL clone appeared to recognize three separate peptide peaks. Preliminary evidence indicates that at least two of these peaks contain related sequence (H. N. Eisen, personal communication).

Similar results concerning peptide dependence in allorecognition have been reported using CTL clones obtained from a K^b mutant (55). Rammensee and coworkers have studied a series of clones of bm1 origin specific for the K^b molecule. An examination of 16 such allospecific clones, in which acid extracted K^b binding peptides were used to reconstitute recognition of RMA-S, indicated that the majority of these clones were peptide dependent in their recognition of K^b. Whereas some clones appeared to recognize a

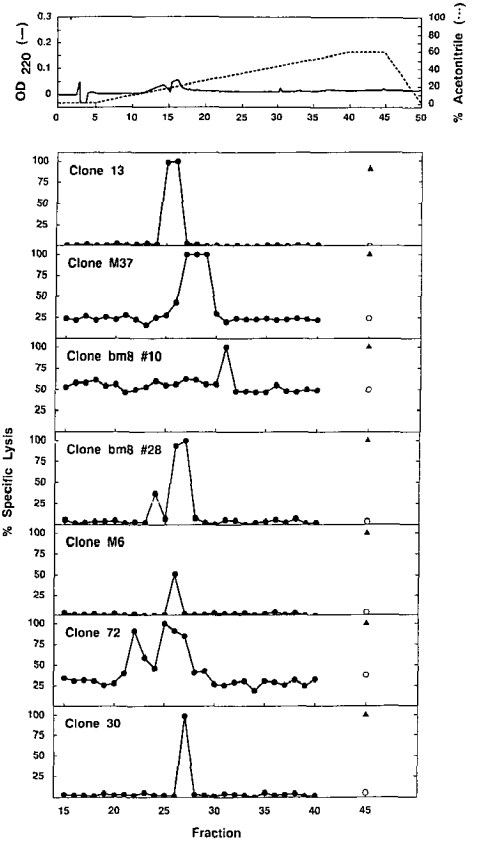

Figure 1 Recognition of reverse-phase HPLC-fractionated K^b-extracted peptides by K^b-specific CTLS. A) Elution profile. B) ^{51}Cr-labeled T_2K^b cells were incubated with individual column fractions and then used as targets for lysis by CTL clones. In each experiment, controls included uncoated T_2K^b cells (○) and EL_4 cells (▲).

unique peptide peak, others were able to recognize two or more peaks or very broad peaks of activity eluting from the HPLC gradient.

The studies referred to above utilized relatively small numbers of alloreactive CTL clones to study the issue of peptide dependence. A set of studies by Englehard and coworkers questioned what proportion of all alloreactive T cells were actually peptide dependent (57, 58). Large numbers of CTL clones were raised against either A2, D^d or L^d and tested for their ability to recognize appropriate T2 transfectants in the absence of exogenously provided peptides.

These studies concluded that 85% of A2 specific clones and 90% of D^d specific clones did not recognize empty class I. Interestingly, a minor

population could be found that recognized both T2 and wild type A2 expressing cells equally well. Such clones are candidates either for recognition of empty class I or recognition of class I plus a peptide produced by an alternative processing pathway that functions in T2 cells (59, 60). The proportion of clones that could recognize L^d molecules on T2 cells was significantly higher (44%). Most likely, however, the majority of these may actually be peptide dependent because they demonstrated species specificity in their recognition of L^d.

CTL Stimulation by Empty Class I

Although the majority of allospecific CTL require peptide to achieve recognition of K^b, considering that these CTL are generated using stimulators that express peptide filled class I, it may be possible to obtain CTL specific for empty class I if the cells used as stimulators express empty molecules. One such study used the T2 cell line in an attempt to address this issue (57). In these studies T2 transfected with either L^d or D^d was unable to stimulate a response specific for either empty or filled L^d or D^d, respectively.

How Peptide Specific Are T Cells?

Several laboratories have recently demonstrated that some alloreactive clones can be reconstituted in their recognition of empty K^b by the presence of noncellular synthetic peptide antigens known to serve as K^b binding antigens. Guimezanes and colleagues (61) demonstrated the ability of an antigenic peptide of the vesicular stomatitis virus nucleoprotein that is normally presented by K^b to sensitize RMA-s cells for recognition by an allo-CTL clone. Bluestone and colleagues (39) obtained analogous results, which suggested that some clones may be peptide dependent but not very peptide specific inasmuch as a variety of different synthetic peptides could promote their recognition of target cells. Indeed, as noted above, some allospecific clones do recognize more than one HPLC peak. This degenerate specificity raises the question of whether the TCR actually binds peptide. The conformational models of T cell recognition of Bluestone (39) and Sherman (18) proposed that the T cell receptor may not actually recognize peptide. Rather, the receptor may be sensitive to the conformation of the MHC that is adapted when a particular peptide is bound. This hypothesis proposes that peptide binding can alter MHC structure sufficiently to permit discrimination by the T cell receptor. According to this theory, two peptides with different sequences may produce a similar conformational change in the molecule, thus resulting in the observation of cross-reactive recognition of unrelated synthetic peptides, as was described above.

An alternative explanation for such recognition is based on affinity considerations. Given that there are thousands of different peptides expressed by each of the different MHC molecules on the cell surface, when a CTL clone is stimulated by a specific endogenous peptide its affinity must be sufficiently high to be triggered through the recognition of very few peptide molecules. As few as several hundred may suffice (62, 63). When mutant targets such as RMA-S or T2 are provided with exogenous peptide, essentially all the class I on the surface presents a single species of peptide. This may represent a 10–1000 fold higher concentration of a specific peptide-MHC complex than is found for the majority of endogenous peptide antigens. Given this unusually high concentration of peptide, it is possible many alloreactive T cells may demonstrate low, yet sufficient affinity for the complex to effect target cell lysis. This explanation is reminiscent of Bevan's affinity hypothesis to explain the high frequency of alloreactive cells (31). Given that, when presented with physiological concentrations of peptide, allospecific clones are highly specific for only one endogenous peptide (as evidenced by both tissue specificity in allorecognition and HPLC resolution of endogenous peptides), the evidence would favor this explanation of an apparent lack of peptide specificity when dealing with high concentrations of synthetic peptides.

Are There Peptide-Independent T Cells?

If the issue of peptide specificity is set aside, the weight of data obtained thus far argues strongly that the majority, and possibly all, alloreactive CTL are peptide dependent in their recognition of class I. Although clones have been reported that recognize class I on mutants such as RMA-S (55, 64) and T2 (54, 57, 58), these represent situations in which some peptide processing and presentation can occur and is responsible for expression of some occupied class I molecules, thereby rendering such data ambiguous in interpretation (59, 60, 65). In contrast, the K^b molecule on T2-K^b reportedly is truly empty (59). When examined for their ability to recognize K^b on T2, most K^b specific clones demonstrated either no recognition or greatly reduced levels of recognition (54). When clones that could recognize T2-K^b in the absence of exogenously provided peptide were further examined, a 10–100 fold enhancement of lysis was observed in the presence of endogenous peptides. Thus such clones may have low affinity for the K^b per se, but their higher affinity for K^b plus peptide may have been responsible for their initial stimulation.

One report suggests the possibility that at least some allospecific T cells may not require endogenous peptide in their recognition of alloantigen. Specifically, Elliot & Eisen reported that they could use class I molecules reconstituted from heavy and light chains to stimulate A2 specific CTL in

a cell free system (66). This study did not rule out the possibility that there may have been residual peptide associated with the heavy chains used for reconstitution. Alternatively, as discussed above, the clone may have had some affinity for empty class I, but it might have had a significantly higher affinity for a specific class I–peptide combination.

In summary, the weight of evidence favors peptide dependence for stimulation and optimal recognition by all allospecific T cells. Although the above considerations have dealt mostly with class I–specific, CD8 CTL, there is now strong evidence that this same conclusion may apply equally to class II–specific alloreactive CD4 T cells.

Self-Peptides Can Also Be Allopeptides

Although each MHC molecule is unique with respect to the types of peptides it may bind, there can be sufficient overlap that the same peptide may bind more than one MHC molecule (67). This raises the interesting question of the ability of a peptide that is able to bind both a self and allo MHC to serve as an allopeptide. Evidence that a self-peptide can, when presented by an allo MHC, be immunogenic has been demonstrated (55, 68). One interesting example was reported by Grandea and Bevan (69) who studied T cell recognition of K^b mutants in which the mutations occurred in residues pointing up from the alpha helix, a position predicted to affect TCR binding but not peptide binding. They found that such mutants were able to stimulate a peptide-dependent alloresponse and that the peptides recognized by these CTL were associated with both wild type and mutant K^b molecules. These results, as well as those of Falk and co-workers (68) represent formal proof of the concept that tolerance of self-antigens (peptides) is MHC restricted.

We have recently observed another situation in which a self-peptide is immunogenic. In the mutant anti-parent combination, bm8 anti K^b, the residues that differ are situated at the base of the antigen binding groove of the K^b molecule and, therefore, should not affect TCR binding to the alpha helices. A CTL clone was identified, bm8#28, that required a peptide found present in both bm8 and the wild type K^b molecule. There was, however, a significant difference in the concentration of this peptide found associated with each of these molecules. It was present at a 10–30 fold higher concentration in the wild type than the bm8 mutant (S. Chattopadhyay, L. A. Sherman, manuscript in preparation). Thus, antigenicity in this situation may be due to a quantitative difference in self-peptide concentration. Bm8 is tolerant to this peptide but apparently only up to a certain density of presentation. The higher concentration at which it may be presented on K^b was immunogenic for bm8 responders. Alter-

natively the same peptide may be presented in a different conformation by the K^b and K^{bm8} molecules.

Which Proteins Contribute the Peptides Recognized by Allospecific T Cells?

The most important consideration in any discussion of the identity of peptides that actually are a part of the alloantigen is the origin of the peptides selected to fill MHC molecules. This issue is dealt with extensively in this particular volume, and the reader is referred to the appropriate sections on antigen processing and endogenous peptides. For the purpose of this discussion, it suffices to consider that peptides present in the antigen binding groove of class I molecules are derived from essentially all proteins synthesized within the cell, whereas those that become associated with class II represent a subset of proteins synthesized by the cell consisting of secretory or membrane proteins and also proteins taken up by the cell into endosomal compartments (70). It may, therefore, be anticipated that peptides representative of proteins that fit into these categories are fair game for allorecognition. As yet, in only a few cases has a peptide recognized by an alloreactive T cell been sequenced and its protein source identified. One such example is an allospecific class II–specific T cell clone (70). Its source protein was identified as a serum component albumin, which is consistent with the types of peptides anticipated for recognition by class II–specific T cells.

Numerous reports have investigated the role of peptides representative of MHC sequences in allorecognition. Initially these peptides were used in studies attempting to identify portions of the MHC molecule directly recognized by T cell receptors (72–74). It soon became apparent that in order to be recognized, the peptide had to be presented by another MHC molecule (75). Based on current knowledge of antigen presentation, it may be anticipated, and has been demonstrated, that MHC molecules represent one source for allopeptides (76, 77, 37). However, the relative contribution of these peptides, as compared with numerous peptides derived from all other-MHC molecules, remains to be assessed.

FUTURE PERSPECTIVES

An important implication of peptide dependence in allorecognition is the fact that tissues that express the same MHC molecules can potentially differ with respect to alloantigen expression due to quantitative and/or qualitative differences in the constellation of peptides presented by their MHC molecules. For example, epithelial cells and lymphocytes both express identical class I molecules, and it is likely that a significant pro-

portion of alloreactive CTL raised to one tissue type will recognize the other. However, due to differences in protein expression by differentiated tissues, it would be anticipated that a proportion of allopeptides will be unique to each cell type. Therefore, T cell tolerance of one type of tissue may not guarantee tolerance of the other tissue. This could be an important consideration in developing strategies to avoid transplant rejection.

Another important lesson learned from current appreciation of the role of endogenous peptide in allorecognition is the fact that alloreactive T cells can be just as antigen-specific as conventional self-MHC restricted T cells. A unique advantage of the allorepertoire is that it can identify a variety of peptides associated with a specific MHC that could not, due to self-tolerance, be efficiently recognized syngeneically. If an allospecific CTL sees a tumor or viral antigen associated with the MHC, than these receptors may, in theory, be useful in specific elimination of tumor or viral infected cells without harming normal cells in the individual.

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THE BIOCHEMISTRY AND CELL BIOLOGY OF ANTIGEN PROCESSING AND PRESENTATION*

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Abstract

T lymphocytes with $\alpha\beta$ receptors recognize antigen in association with the polymorphic products of the class I and class II loci of the major histocompatibility complex (MHC). This presentation of antigen results from the intracellular generation of protein fragments, and the binding and transport to the cell surface of these peptides in stable association with the MHC class I and class II molecules. Each class of MHC molecule appears specialized for capture of peptides present in a particular intracellular compartment. We describe here the structural basis of peptide-MHC molecule interaction, the differences in biochemical behavior that focus the two classes of MHC molecules on peptides of distinct size and location, and the cell biology of MHC molecule transport, peptide generation, and intracellular movement. The importance of con-

formational changes accompanying peptide binding that affect subunit stability of MHC molecules, and the relationship between these changes and the handling of proteins by intracellular chaperones, are emphasized as key features in the operation of the class I and class II presentation pathways.

INTRODUCTION

The specificity that characterizes adaptive immunity derives from the clonal reactivity patterns of B and T lymphocytes. In the 1960s and 1970s, it became clear that T cells showed a completely distinct pattern of antigen reactivity from that shown by the surface-bound antibodies of B cells. Responses seemed restricted to proteins, direct antigen binding was difficult to demonstrate, and most remarkably, T cell responses were limited by and restricted to allelic forms of a set of highly polymorphic glycoproteins encoded in the major histocompatibility complex (MHC) (1–6).

A biochemical explanation for these features of T cell antigen specificity emerged in the 1980s. The key findings were that: (i) the so-called "dual specificity" of T cells for antigen and MHC molecules was carried by a single receptor structure (the $\alpha\beta$ TCR) (7–9), and (ii) the MHC molecules controlling T cell reactivity were peptide binding proteins that presented a fragment of the original protein antigen bound tightly in their polymorphic region (10–12). This paradigm of antigen recognition of peptide displayed on the cell surface in association with class I or class II molecules makes eminent sense for the immune system. All pathogens, and many transformed/malignant cells, possess proteins that are different than those typically expressed by normal host cells. Thus, the unique peptides from such proteins can serve as flags for the presence of infection or transformation.

But this model raised new questions concerning the mechanisms by which allelic polymorphism controlled antigen presentation and recognition, why two distinct classes of MHC molecules with different gene and protein domain organization have evolved, and what intracellular events and properties of the two classes of MHC molecules accounted for the generation and capture of peptide antigens. Advances in our knowledge of the structure of MHC molecules (13–19) and a large number of peptide and MHC mutant structure-function studies (20–25) have provided an increasingly detailed picture of how allelic variation influences antigen presentation. The answer to the second question seems to be that each of the two classes of MHC molecules focuses on capture and presentation of peptides in distinct intracellular locations (26–29). Class I molecules appear committed to peptides present in the endoplasmic reticulum (ER) that derive from proteins actively synthesized by the cell or entering the cytosol

(30, 31). This allows MHC class I–restricted cytotoxic $CD8^+$ T cells to focus their effector function on cells that are potentially harmful to the rest of the body, whether by release of additional infectious organisms or by malignant growth. In contrast, class II molecules seem concerned primarily with peptides reaching the endocytic pathway, most often from exogenous protein sources (32, 33). This permits regulatory $CD4^+$ T cells to be elicited without requiring direct interaction with individual infected cells, while at the same time providing a mechanism for detecting cells with intracellular parasites confined within endocytic organelles. The answer to the third question lies in differences in class I and class II protein biochemical behavior (34–36) and intracellular handling (37, 38).

This review emphasizes this relationship between protein structure and biological activity, attempting to show how an understanding of protein folding events within the cell, and of the operation of the machinery for handling properly and improperly folded proteins, can explain many of the functional properties of MHC molecules.

STRUCTURAL ORGANIZATION OF MHC CLASS I AND CLASS II MOLECULES

Class I

MHC class I molecules consist of a polymorphic type I integral membrane glycoprotein heavy chain of about 46 kDa, noncovalently associated with a 12 kDa soluble subunit, β2-microglobulin (β2m) (39). The heavy chain consists of two distinct extracellular structural units—the membrane distal, peptide binding region formed by the intimate association of the α1 and α2 domains, and the membrane proximal, CD8-binding portion termed α3. Class I heavy chains possess separate exons that each encode a distinct domain of the protein (40–42). β2m is a single, compact immunoglobulin-like domain that lacks a membrane anchor and exists either associated with the class I heavy chain or free in plasma and tissue fluids.

High resolution x-ray crystallographic structures have been derived for three human class I molecules (HLA-A2, HLA-A68, and HLA-B27) and more recently for the mouse $H\text{–}2K^b$ molecule (13–18, 21, 42a). Each represents the extracellular portion of the molecule, complexed to either an apparently heterogenous selection of self-peptides in the case of HLA-A2 and HLA-A68, a somewhat more homogeneous set of self-peptides in the case of HLA-B27, or to homogeneous synthetic peptides for the two $H\text{–}2K^b$/peptide complexes. The most striking aspect of each of these structures is that the $\alpha1\alpha2$ domain unit forms a single peptide binding site supported by a β-pleated sheet floor containing eight strands and bounded by two α helices, one from α1 and one from α2. β2m makes contact not

only with the immunoglobulin-like α3 domain, but also with the β sheet floor of the α1α2 peptide binding region. A more detailed examination of the first two human HLA-A crystal structures revealed the presence of a set of pockets, in some cases extending deep between the floor and helical walls of the binding domain (14). A similar distribution of pockets exists in each class I molecule examined to date, and these have now been designated A through F (14, 16).

Class II

MHC class II molecules are type I heterodimeric integral membrane proteins (43). Each dimer consists of one α and one β chain in noncovalent association. As with class I, the intron-exon organization of genes encoding class II α and β chains corresponds to functional domains in the protein molecule, with the second exon containing coding information for the bulk of the positions at which extensive intraspecies polymorphism exists (44, 45). Exon 4 gives rise to the connecting regions and well-conserved transmembrane segments. Exons 5 in the α chain and exons 5 and 6 in β encode various conserved portions of the cytoplasmic tails.

No crystallographic analysis of class II molecules was available at the time this review was written, but three lines of evidence support the view that the structure of MHC class II molecules closely resembles that of class I molecules. First, circular dichroism studies show a similar content of α helix (46). Second, mutagenesis-based structure-function studies show a remarkable parallel with similar studies on class I (22). Third, modeling by protein homology suggests a very similar structure in the peptide binding domain (22), although differences that are likely to contribute significantly to distinct peptide binding properties clearly exist. Two known differences are the assembly of the class II peptide binding region from noncovalently associated polypeptide chains and the transmembrane anchoring of both class II subunits, compared to only the heavy chain of class I. This latter feature may contribute to the apparently greater stability of class II molecules as compared to class I molecules.

SELECTIVITY IN MHC BINDING OF PEPTIDES

Class I

POLYMORPHISM AND ANTIGEN-SPECIFIC PRESENTATION The most striking characteristic of the MHC class I molecules is their profound polymorphism (47). Early functional studies using only T cell assays were limited in their ability to determine the precise mechanism(s) by which this polymorphism affected antigen presentation and recognition. The determination of the sequences of peptides eluted from class I molecules

and of the crystal structure of several class I molecules has provided the information needed to interpret these early studies and has yielded a general model for the control of peptide presentation by class I molecules.

PEPTIDES BOUND BY CLASS I MOLECULES Evidence that bound peptides co-purified with class I molecules came from the recognition of electron density in the maps of HLA-A2 structures that could not be accounted for by the known amino acid sequence of the HLA-A2 heavy chain (13). This led to development of immunoaffinity isolation and acid elution methods for studying the peptides naturally loaded into class I molecules. Van Bleek & Nathenson (48) recovered radiolabelled peptide from isolated H–2K^b molecules of cells infected with vesicular stomatitis virus (VSV). Sequencing indicated an octamer with tyrosines at positions 3 and 5 and leucine at position 8, corresponding to the core sequence RGYVYQGL in synthetic peptides capable of sensitizing cells for recognition by VSV specific, H–2K^b-restricted CTL. Rötzschke et al (49) used HPLC fractionation, bioassays, and synthetic peptides to identify a naturally processed influenza hemagglutinin determinant, which corresponded to a nonamer within a previously identified sequence. Important general characteristics of self-peptides bound to class I were identified by Falk et al (50), who subjected pools of peptides eluted from single class I alleles to Edman degradation. Although unique sequences were not obtained from these heterogeneous mixes, particular amino acids gave very strong signals at specific positions, and the sequencing signals did not usually extend beyond position 9. The predominant residues could be found in comparable positions in most peptides previously identified as being presented by the relevant class I molecule, giving rise to the concept of "motif" or "anchor" amino acids important in promoting binding to a particular allele of class I. The lack of strong sequencing signals beyond 9 amino acids in these pools of eluted material fit well with the 8 and 9 residue length of the specific viral peptides found associated with class I molecules, suggesting that length was important for tight peptide association. The occurrence of strong signals at a given position in the sequence indicated that the NH_2-termini were a fixed number of residues from the anchor positions in each peptide.

Several groups have used related strategies to identify and characterize class I–associated peptides. Jardetzky et al (51) sequenced HPLC purified single peptide peaks, revealing the HLA-B27 motif of arginine at position 2 and a preference for a basic residue at the COOH-terminal ninth position. Hunt et al (52) applied microcapillary HPLC and electrospray ionization–tandem mass spectrometry to evaluate both the complexity of HLA-A2 bound peptides and then their sequence. This methodology permitted these

investigators to quantitate as many as 200 different peptides co-purifying with HLA-A2. More recently, the motifs of several other MHC class I–bound peptides have been determined (19, 53, 54). Of the peptides whose source protein could be unambiguously identified by this approach, all but one (55, 56) are from abundant cytoplasmic or nuclear proteins (51). This methodology and the predictive potential of motif patterns can now be used to determine the naturally processed and presented peptides from infectious organisms or those self-peptides involved in autoimmune disease.

MOLECULAR BASIS FOR THE GENERAL AND ALLELE-SPECIFIC CHARACTERISTICS OF CLASS I PEPTIDE BINDING The relatively homogenous nature of the peptide visualized in HLA-B27 electron density maps permitted detailed model building of a prototype bound peptide (15). The consensus peptide was nine residues long and in an extended conformation with a central kink. The recent determination of the structure of either a VSV-derived octamer or a Sendai virus (SEV)-derived nonamer bound to H–2K^b (17, 18, 42a) confirmed these general features and provided additional details about how individual peptides are bound by the same class I molecule. Both the HLA-B27 and H–2K^b analyses indicate that a critical aspect of peptide binding to class I is the direct formation of hydrogen bonds to the peptide's NH_2-terminal amine through conserved MHC residues tyrosine 7, tyrosine 59, and tyrosine 171, as well as extensive hydrogen bonding to the terminal carboxyl group from class I tyrosine 84, threonine 143, and lysine 146. Additional hydrogen bonds are made to the carbonyl groups of the first and penultimate amino acid residues by tyrosine 159 and tryptophan 147, respectively. Such conserved bonds to features common to all peptides suggest how otherwise polymorphic class I molecules can each act as an effective peptide binding protein for a wide diversity of peptide sequences. The critical contribution of these conserved bonds to class I peptide interaction also accounts for the stringent length restriction on class I–associated peptides observed in the eluted peptide pools, as peptides substantially longer or shorter than the 8–10 residue optimum cannot readily make these bonds and still fit snugly in the remainder of the binding region.

The second striking feature of the class I–peptide interaction revealed by these studies is the contribution of allelic polymorphism to the shape and chemical character of the pockets within the binding region and the role of these features in discriminating among peptides of similar length but differing sequence. Almost all the side chains of the polymorphic amino acids in the binding domain are oriented inward toward the region containing peptide (13–18, 21). This suggests that the major role of poly-

morphism is to regulate the binding of peptides, rather than to directly affect interaction with the T cell receptor. The bulk of these polymorphic positions contribute to variations in the shape and chemical nature of smaller pockets within the binding domain. Garrett et al (14) suggested that the B pocket of HLA-A2 had a hydrophobic character well-suited to binding of the leucine residue at position 2 of known HLA-A2-presented peptides. The modeling of bound peptide in HLA-B27 (15) and the sequences of numerous HLA-B27–associated peptides (51) clearly revealed the chemical compatibility of an aspartic acid in the HLA-B27 B pocket with the arginine anchor residue at position 2 of the peptides. The mouse H–2K^b structures confirm this general principle of pocket polymorphism with its relationship to interaction with motif residues, showing how the depth of pocket C in H–2K^b allows it to accommodate the bulky peptide anchors of phenylalanine or tyrosine at position 5.

The comparison of the nonamer- and octamer-bound H–2K^b structures also shows how a kink in the longer peptide allows its accommodation in the H–2K^b groove with proper bonding to the conserved residues in the A and F pockets. Finally, the two H–2K^b structures reveal that the fine structure of a single class I molecule can vary significantly depending upon the peptide bound (most remarkably with respect to the orientation of arginine 155, a residue potentially accessible to the T cell receptor). This structural picture of the roles of conserved and polymorphic positions within the $\alpha 1\alpha 2$ superdomain in selective peptide binding and contributions of the MHC class I molecule and peptide to a complex surface available for T cell receptor interaction is consistent with a large literature on the effects of mutation in this region on antigen presentation to class I–restricted T cells (20, 24, 25, 57–60).

More precise mutagenesis-based mapping has been based on the description of the binding pockets visualized crystallographically. Latron et al (61) focused on the MHC residues modeled to interact with the NH_2-terminal amine (in the A pocket) and the carboxyl terminus (in the F pocket). Size-conservative mutations lacking the ability to hydrogen bond to the peptide were made at positions 7 or 171 (A pocket) and 84 or 143 (F pocket). Either change in the A pocket residues decreased by two orders of magnitude the half maximal ability of peptide to sensitize targets for CTL lysis, while changes in the F pocket residues had little or no effect. Unfortunately, direct peptide binding data were not reported, so a clear distinction between loss of peptide binding vs alteration of the conformation of the peptide-class I complexes could not be made. The apparent absence of a crucial role for some of the conserved residues in the F pocket contrasts with the findings of Elliott et al (62) who observed that both COOH-terminal amidation as well as NH_2-terminal acylation of an

H–2K^b–restricted peptide were required to fully eliminate peptide binding.

CLASS Ib MOLECULES HAVE A UNIQUE PEPTIDE BINDING PREFERENCE A much debated question has been whether genes that encode "nonclassical" class I molecules, now known as the class Ib molecules, have any biological function. T cell recognition of class Ib molecules encoded in the H–2T region have been reported (63–67), but such observations did not provide clear evidence for physiological function. More recently, it has become apparent that the maternally transmitted antigen, "Mta," which is encoded by a polymorphic mitochondrial gene for a subunit of NADH dehydrogenase, is presented to CTL by the class Ib protein encoded by the H–2M3 gene, formerly known as Hmt (68, 69). A unique feature of H–2M3 is that it binds peptides containing the N-formyl group found in the methionine initiating bacterial and mitochondrial proteins (70). H–2M3 has phenylalanine substituted for the highly conserved residue tyrosine 171 (71), which may be critical to the ability of H–2M3 to accommodate an N-formyl group. The immunological value of an MHC molecule that can present N-formylated peptides is apparent from reports that peptides from the intracellular bacterial pathogen, *Listeria monocytogenes*, are presented by H–2M3 (72, 73).

Class II

ALLELIC POLYMORPHISM AND PEPTIDE BINDING Extensive intraspecies polymorphism is also a hallmark of MHC class II molecules. Both function-based and direct-binding assays have shown that this polymorphism plays a major role in determining which peptides show long-lived binding to class II molecules (74). Immune response (IR) gene control usually reflects the differential capacity of allelic MHC products successfully to capture and present to T cells a suitable peptide derived from the antigen (74–77). Residues presumed to lie in both the floor of the binding site and those pointing in from the helices can modify the specificity of peptide capture. Single residue changes can decrease binding of certain peptides by several orders of magnitude (77, 79). Not all variations in MHC molecule structure result in quantitative changes in this interaction. Despite a similar level of bound peptide, some T cells show nearly an all-or-none difference in their response to the peptide associated with wild-type vs mutant class II molecules (77–80). These data emphasize that the linear sequence of an antigenic peptide limits but does not define its epitopic structure—this is determined only upon binding to a particular MHC molecule.

Presentation of bacterial superantigens (e.g. SEB, SEA, TSST–1) depends on physical interaction with class II molecules (81–83) but differs

in many respects from binding of peptides. Although some influence of allelic polymorphism can be seen in binding of these proteins (84–88), such polymorphism plays a lesser role than in presentation of peptides. Furthermore, it appears that such proteins bind primarily to regions outside of the peptide binding groove occupied by antigenic peptides (89, 90). Different bacterial toxins are influenced in their binding by distinct regions of the class II molecule (91, 92).

NATURALLY PROCESSED PEPTIDES ASSOCIATED WITH CLASS II MOLECULES

Peptides have been eluted from immunoaffinity-purified class II molecules and analyzed by mass spectroscopy or Edman degradation (93–96). Such analyses have shown that each MHC allele gives a characteristic profile of eluted peptides, consistent with the role of allelic polymorphism in "determinant selection." In contrast to the situation with class I, class II–bound peptides are longer and more heterogeneous in size, ranging in length from 12 to more than 20 residues. This heterogeneity is not simply the result of differing lengths for peptides from distinct proteins; it also reflects the capture of nested peptide sets from a single protein. Because both N and C terminal extensions are observed, these data suggest that class II lacks a requirement for the relatively buried conserved N and C terminal bonds contributing to class I–peptide interaction and may have a more "open" structure that allows these longer peptides to lie in the binding groove without substantial kinking. Why the presumably protruding ends would not be trimmed "flush" with the binding site by exopeptidases in the antigen processing compartment is an intriguing question.

Because class II–associated peptides with common core sequences show staggered NH_2-termini, it is not possible to carry out sequencing of mixed pools of eluted peptides to identify motifs or anchor residues. Instead, distinct peptide species have been sequenced after HPLC or mass spectroscopic isolation, and the sequences aligned by sliding the core regions of the overlapping sequences with respect to one another (94–96). This method has allowed identification of motifs for binding to particular class II alleles. These generally involve only 2–3 residues from among a core region of 7–9 residues, consistent with studies using synthetic peptides (97–99). The motif residues are presumed to occupy polymorphic pockets of the type identified in the class I binding site.

The sources of peptides bound to class II molecules fit with the general model of class II function and the class II processing pathway, in that the predominant identifiable species are derived from proteins with ready access to the endocytic pathway (93–96). Only a single example of a peptide from a highly abundant cytoplasmic protein has been found, and the basic

charge of the source molecule suggests that it may have come from released debris efficiently endocytosed in association with anionic surface proteins (100). This implies that such truly "endogenous" proteins access the class II pathway inefficiently compared to the class I pathway. However, very few of the less abundant peptides associated with class II have been analyzed, and these might prove to come in part from such proteins.

MHC CLASS I AND CLASS II SYNTHESIS AND EXPRESSION

Class I

THE BASIC PATTERN OF CLASS I BIOSYNTHESIS Class I heavy chain and β2m are both type I proteins and enter the endoplasmic reticulum (ER) via the signal-recognition and transport apparatus. The heavy chain associates rapidly with an ER resident protein termed p88 or Ip90 (37, 101, 102). This molecule is a Ca^{++} sensitive protein called calnexin that is associated with the signal sequence-dependent translocation mechanism (103). Ip90 has been reported to associate with a variety of membrane proteins in the ER, including components of the T cell and immunoglobulin receptors (102). Preliminary data suggest that the transmembrane portion of class I may contribute to interaction with p88 (D. Williams, personal communication), a conclusion consistent with the involvement of p88 in regulating expression of multimeric protein assemblies with critical interactions in the transmembrane region. This raises the intriguing possibility that class I does not exist as a simple monomer, but undergoes either some form of homo-oligomerization or protein-protein interaction as part of its maturation. Class I heavy chains can form dimers in liposomes and on cells (104) and tetramers on cell surfaces (105). Whether these higher order forms are related to maturational release from p88 remains to be determined.

In cells lacking co-expression of β2m, the large majority of class I heavy chains remain associated in the ER with p88 until degraded. In normal cells, after heavy chain translation is complete, β2m associates with the heavy chain-p88 complexes, but this interaction does not result in rapid dissociation from p88 (37). Thus, β2m is necessary but not sufficient for release from p88. Consistent with the β2m-dependence for release from p88 ER retention, numerous studies have shown the critical role of β2m in achieving significant expression of class I on the cell surface (106, 107). However, the requirement for β2m in class I transport is not absolute. A low level of certain class I heavy chains can be detected on the surface of cells lacking β2m expression (107, 107a,b). Most or all of these molecules

lack numerous serological epitopes characteristic of the same class I expressed in association with β2m, suggesting that an alternative folding state of a subset of class I heavy chains allows them to bypass the ER retention mechanism and reach the cell membrane in small amounts. Release from p88 in the presence of β2m appears to occur within the ER itself without movement to the *cis*-Golgi network (intermediate compartment) (37). Following exit from the ER, class I heavy chain β2m complexes move through the Golgi stacks to the trans-Golgi network, and then to the cell surface by the default secretory pathway (108).

Class II

THE BASIC PATTERN OF CLASS II BIOSYNTHESIS The α and β subunits of MHC class II molecules are typical type I membrane glycoproteins and can be found associated within two minutes of synthesis (109). This assembly appears to require certain conserved features of the transmembrane regions of these polypeptides (110). Under physiological circumstances, the α and β chains do not co-assemble alone, but in the presence of invariant chain (Ii) (111), which is a nonpolymorphic type II membrane glycoprotein (NH_2-terminus in the cytoplasm, COOH-terminus in the ER lumen) (112, 113). Ii rapidly forms noncovalently associated trimers in the ER (114), and class II $\alpha\beta$ dimers either quickly associate with these trimers or may assemble on the trimers themselves (115). Ii is not a single protein, but a family of proteins generated by alternative splicing and in human, but not mouse, by alternative translational initiation (116–118). Thus, in human there are four primary forms of Ii, called p31, p33, p41, and p43 [some investigators refer to these species as p33, p35, p43, and p45, respectively]. The p31 and p33 differ by 17 residues at the NH_2-terminus in the cytoplasm as a result of differential translation initiation, as do p41 and p43. The p31 vs p41 and p33 vs p43 forms differ by an alternatively spliced exon that adds a thyroglobulin-like domain to the protein.

After assembly of the $\alpha\beta$Ii complexes, these multimeric assemblies move out of the ER and through the Golgi stacks, undergoing formation of complex N- (and for Ii, O-) linked glycans and terminal sialation (119, 120). These events take place within 30–60 min of initial class II biosynthesis. When the $\alpha\beta$Ii complexes reach the trans-Golgi, they deviate from the typical exocytic pathway that takes most proteins, including class I molecules, directly to the cell membrane, and instead they move to the endocytic pathway (108). They reside there for several (2 to 6) hr, after which class II dimers reach the surface free of Ii. Ii is removed from class II after egress from the trans-Golgi via a process that appears to involve sequential NH_2-terminal proteolytic cleavages (121, 122). The removal of

Ii from class II can be inhibited by agents that interfere with the function of acid proteases such as leupeptin (an inhibitor of cathepsin B) (121, 123) and chloroquine (124), which raises endosomal/lysosomal pH.

THE INTRACELLULAR TRANSPORT AND LOCALIZATION OF Ii AND MHC CLASS II MOLECULES This deviation of class II molecules from the default secretory route and the obvious importance of this phase of class II intracellular transport in antigen presentation has prompted careful study of class II localization and of class II movement within the cell.

Steady-state distribution of class II molecules In transformed B cells (125, 126) and in activated macrophages (127), class II molecules can be detected in the ER, Golgi stacks, trans-Golgi region, on the plasma membrane, and in intracellular vesicles. In some studies, these vesicles were characterized as early endosomes (125), whereas in others, they were considered a unique compartment ultrastructurally and histochemically related to lysosomes (126). In macrophages, most of the intracellular class II was found in submembranous sacs lacking markers of late endosomes or lysosomes (127). The distribution of class II is clearly different in cells of distinct tissue type (128) and may even vary among cell lines of nominally identical histological origin. In addition, the definitions and markers used to identify subcompartments in the endocytic pathway come from studies in a limited set of prototypic cells and may not apply precisely to the endosomal/lysosomal organelles in other cells.

Invariant chain distribution In some class II gene-transfected cells lacking co-expression of Ii, class II appears to move directly from the trans-Golgi to the surface membrane (129, 130). This difference from the observations in cells co-expressing class II and Ii prompted several groups to study the intracellular distribution of Ii itself and to examine this molecule for signals mediating movement from the ER to the endosomal pathway.

The human Iip33 form is retained in the ER (129, 131). The NH_2-terminal segment of the Iip33 cytoplasmic tail shares with the adenovirus E19 protein a motif of basic amino acids that mediates ER retention (132, 133). Ii is usually produced in excess of class II in normal cells (120, 122), and the association of p31 or p41 with p33 or p43 in trimers and higher molecular weight aggregates may contribute to retention of the p31 and p41 forms in the ER, where they undergo degradation to smaller, NH_2-terminally cleaved forms (114, 122, 134).

In contrast, when not complexed with p33 or p43, varying proportions (5–20%) of the p31 form of human Ii, and the p31 and p41 forms of mouse Ii move through the Golgi complex and accumulate in vesicular structures (129, 130, 135). This localization depends on signals within the 30 residue

NH_2-terminal cytoplasmic tail, as deletion of 15 or more residues results in movement to the cell surface (129, 130, 135, 136). These same truncations also increase the proportion of Ii showing acquisition of endoH resistant glycans, suggesting that this region of Ii plays a role in retarding exit of Ii from the ER even when the E19-related retention signal is absent.

At high levels of Ii expression, Ii is found predominantly in an unusual cohort of intracellular vesicles [large vesicular structures (LVS) or macrosomes] (130, 137). These macrosomes are accessible to transferrin as are early endosomes, they are much larger than typical endocytic vesicles, and they are not present in cells lacking Ii, indicating that they are generated by an active process dependent on the presence of Ii. The most remarkable feature of these macrosomes is that they alter the rate of endocytic flow from the early endosomal sorting compartment to later endosomes and lysosomes. The extent of macrosome formation seems to correlate with the level of free (non-class II associated) Ii that leaves the ER. Taken as a whole, these findings suggest that Ii can regulate the rate of distal movement of endocytosed antigen and of newly synthesized class II-Ii complexes arriving from the trans-Golgi. Although the significance of these observations is unclear at present, one possibility is that this effect of Ii enhances endosomal fusion and mixing so that incoming antigen and newly synthesized class II-Ii complexes will reside in the same maturing endocytic vesicle, rather than coming to occupy discrete vesicles. This might contribute to the efficiency of peptide capture by class II when antigen is limiting.

Chaperone and transport function of Ii complexed with class II Co-expression of class II with Ii alters both the pattern of intracellular localization and the biochemistry of protein maturation of the two molecules. Based on experiments in Xenopus oocytes, Claesson-Welch & Peterson (138) first suggested a role for Ii in "chaperoning" class II from the early biosynthetic compartment. Subsequent experiments (139, 140) demonstrated class II surface expression by Ii-negative cells, making it unlikely that Ii had an obligate role in this process. However, Layet and Germain (141) confirmed the conclusions of Claesson-Welch and Peterson; they first showed in mammalian cells that Ii could augment class II surface expression, at least in part by improving class II egress from the ER. These results have been confirmed and extended by others (142, 143). Similarly, Ii in the presence of class II moves more efficiently from the ER to Golgi (129, 144), indicating that transport is mutually facilitated by class II-Ii co-assembly. It is likely that Ii-class II interaction prevents association of these molecules with chaperones that are known to bind to and retain misfolded or partially folded proteins in the ER (145, 146). Whether this

reflects a steric effect of chain association that hides chaperone recognition sites or a more correct folding of Ii, α and β chains as they associate is not yet clear. This latter model is consistent with data showing that class II molecules have distinct pattems of reactivity with monoclonal anti-class II antibodies when produced in the presence or absence of Ii (147).

Movement of class II beyond the trans-Golgi has been studied in cells co-expressing Ii physiologically or as a result of transfection. Cresswell (148) reported that the sialic acid residues of recently synthesized class II molecules and intact associated Ii were cleaved by neuraminidase confined to the early endocytic compartment by conjugation to transferrin. This implies that such recently formed complexes at least transit through early endosomes prior to Ii degradation and removal. Related studies also found that newly synthesized class II was accessible to endocytosed neuraminidase prior to expression at the cell surface (149). Transfection experiments showed that the co-expression of Ii with class II results in the appearance of substantial amounts of class II in early (130, 131) and/or late (129) endocytic compartments. Taken together, these data suggest that class II-Ii complexes move from the trans-Golgi to early endosomes, then accumulate primarily in a late endosomal/prelysosomal compartment distinct from terminal (dense) lysosomes, which show virtually no detectable class II content (126, 127, 150).

The mechanism of transport from trans-Golgi to early endosome is not known. Experiments to date have not ruled out movement to the cell surface, followed by very rapid internalization into endosomes. A direct route from the trans-Golgi to early endosomes without transient expression on the plasma membrane has been demonstrated for cathepsin D (151). However, class II does not co-localize with mannose–6-phosphate receptors in the trans-Golgi (126) and does not co-distribute with γ adaptin-rich regions of the trans-Golgi where mannose–6-phosphate receptors accumulate (130). These data raise the possibility that class II transport to early endosomes may involve either a distinct route (e.g. via the plasma membrane) or a different vesicular vehicle, perhaps a non-clathrin coated vesicle of the type that mediates movement between Golgi stacks (152).

Ii in early endosomes seems largely intact even though significant cathepsin B activity has been observed in such vesicles (153). The possibility remains that a fraction of Ii is completely degraded at this site by proteases including cathepsin B, freeing a cohort of class II to capture peptide and move to the cell surface. Nevertheless, the bulk of the initial C-terminal cleavage of Ii appears to occur during transport to or in later parts of the endocytic pathway, possibly in late endosomes or prelysosomes (130). Leupeptin inhibits late cleavage events associated with Ii removal from class II molecules, and complexes of class II with a fragment of Ii accumu-

late in cells treated with this drug (121). Such complexes remain trapped within the cell (149; R. Germain, C. Layet, unpublished observations), accumulating in vesicles with the characteristics of late endosomes/prelysosomes (130). Thus, Ii not only affects the movement of class II within the cell from the early biosynthetic compartment to the endosomal pathway, but also the release of class II from the endosomal pathway for movement to the cell surface. This trapping function of partially degraded Ii may account for the site of predominant class II accumulation within cells.

In some cells lacking detectable Ii, class II is found in cytoplasmic vesicles (154). Available data does not establish whether such molecules are recently synthesized and in the process of moving to the cell membrane, or whether they have moved into the endocytic pathway from the cell surface and are awaiting degradation and destruction. Similarly, some Ii has been detected on the surface of B cells (155, 156) and on freshly isolated Langerhans cells (157, 158), but whether it reaches the surface with class II or represents some (mis)-sorting of free Ii is unknown.

CONFORMATIONAL CHANGES IN MHC MOLECULES

Class I

CHANGES IN STRUCTURE AND ASSEMBLY ACCOMPANYING PEPTIDE BINDING BY MHC CLASS I MOLECULES Despite their contribution to our detailed understanding of the chemical basis for MHC class I binding of peptides, the static crystal structures failed to reveal a critical aspect of the biochemical properties of MHC class I molecules essential to understanding their biology. A more complete understanding of the dynamic behavior of the class I molecule in interaction with peptide and β2m only developed from the recognition that antigenic peptide could induce a new conformation of the heavy chain and stabilize heavy chain–β2m complexes (34). These breakthrough experiments involved studies of antigen and peptide presentation by a mutant mouse lymphoma cell (RMA-S) that synthesized normal amounts of class I heavy chains and β2m, but had only low steady state surface expression of class I molecules. These cells were defective in presentation of antigen produced during active viral infection but were remarkably effective in presenting synthetic peptide, despite their initial low level of class I expression. The addition of antigenic peptide was found to markedly increase the surface expression of the class I allele known to present the peptide used, and after detergent lysis, such peptide-treated cells showed a significant increase in stably assembled heavy chain-β2m complexes reactive with $\alpha 1\alpha 2$ conformation-specific anti-

bodies. Although this increase in assembled and properly folded class I molecules was later shown to result from peptide binding after cell lysis, rather than peptide entry into the cells during culture (159), the authors correctly concluded that peptide was promoting assembly of heavy chains and β2m in a native conformation. They also suggested that RMA-S showed poor expression of class I due to a defect in supplying the peptides needed for such assembly to the class I heavy chains and β2m in the ER.

The observations made with RMA-S have provided the framework for a large number of subsequent studies that have given additional insight into the relationship among peptide, β2m, and heavy chain conformational change in the generation of functional class I molecules expressed at the cell surface for T cell recognition. The ability of peptides known to bind to particular class I alleles to increase surface expression on cells with high level synthesis but low surface expression has been confirmed for numerous mouse and human molecules, using both mutant cells deficient in their ability to present naturally processed antigens and normal cells that appear unable to provide adequate peptide to fully load certain class I alleles such as H–2L^d (160–163).

Because the peptide-induced increase is blocked by the drug brefeldin A and other inhibitors of protein export, it appears that the increase is due to the preservation on the cell surface of molecules that reach the cell membrane and are rapidly cleared (34, 164, 165). Ljunggren et al (166) provided a clear demonstration of this in showing that the mutant RMA-S cells have enhanced surface class I expression when cultured at room temperature. The molecules that accumulated on such cells were rapidly lost to serologic detection after the temperature was raised to 37°C, and in cell lysates, the class I-β2m complexes that accumulated at room temperature rapidly denatured when exposed to 37°C. These data were interpreted to indicate that the class I-β2m association was weak in the absence of proper peptide binding, but enhanced at low temperature, allowing prolonged surface residence. Increased temperature resulted in the rapid dissociation of the complexes and heavy chain denaturation. Whether the weaker associations of heavy chain and β2m were due to the complete absence of associated peptide or to the availability only of peptides with poor capacity to stabilize the complexes is an issue that has yet to be resolved.

More recent studies have extended these initial observations. Ortiz-Navarrete & Hämmerling (167) have reported that antibodies to conformational sites involving the α1 and α2 domains of class I also could lead to accumulation of class I on the surface of RMA-S, presumably substituting for peptide in maintaining the stability of loosely associated heavy chain-β2m complexes reaching the cell membrane. Both Elliott et

al (168) and Schumacher et al (169) noted that stabilization of the class I heavy chain-β2m complex was optimal with short peptides identical or similar in length and sequence to those found naturally associated with class I isolated from intact infected cells. These data indicated that very efficient selection of optimal peptides was occurring in the cell, and that the ability of a peptide to stabilize the class I structure was a key feature in selecting peptides for presentation to T cells. This ability of class I molecules to select short peptides of an appropriate length and composition has been graphically demonstrated by Schumacher et al (170) who exposed RMA-S cells to radiolabelled synthetic peptide libraries and then analyzed the spectrum of peptides bound by H–2K^b and H–2D^b, and also in studies of the important role of serum proteases in trimming longer synthetic peptides to optimal ligands for class I presentation (171, 172)

Additional insight into the mechanistic aspects of class I peptide binding came from studies on the requirements for presentation of peptide antigen by cells or purified class I molecules. Several groups observed that free β2m was necessary to achieve effective presentation of such exogenously added peptides (173–177). These results were a counterpoint to the data on the role of peptide in class I structure, suggesting that peptide capture required β2m to be available for binding to the heavy chain, just as stable β2m binding to the heavy chain required peptide. From these and other studies using either analysis of class I molecules in cellfree lysates or on cell surfaces, the concept arose of equilibrium reactions among the three components of the mature class I trimer (heavy chain, β2m, and peptide) during assembly (159, 165, 168, 178–180). β2m was needed to stabilize the peptide-heavy chain interaction, and peptide stabilized the β2m-heavy chain interaction. Both binary reactions have been demonstrated, but the apparent lifetime of the binary pairs is low compared to the trimeric complex. Thus, peptide can conform class I heavy chains in the absence of β2m (168), but this requires very high peptide concentrations and the resulting molecules are highly unstable. Conversely, high concentrations of free β2m can preserve class I folding (though it is unclear whether this can happen in the complete absence of peptide), but again, the structure is relatively unstable (165, 179). Peptide and β2m synergize in forming a long-lived, properly folded complex.

Other investigators have used cell-free translation systems or the assembly of denatured heavy chains and β2m to further explore the relationships among class I heavy chain, β2m, and peptide in creating stable class I molecules. Kvist & Hamann (181) studied HLA-B27 and human β2m derived from synthetic mRNA translated in a reticulocyte lysate system coupled to a human cell microsomal post-translational processing system. They observed that availability of an antigenic peptide

was required for promoting the assembly of the HLA-B27/human β2m heterodimer. In a later study, using microsomes derived from cells lacking β2m, they demonstrated a dependence on both β2m and peptide for promoting assembly (182). Ribaudo & Margulies (183) used a rabbit reticulocyte translation system coupled to a canine pancreas microsome processing system to study the mouse class I molecule H–2L^d and mouse β2m. Available monoclonal antibodies permitted the discrimination of $\alpha1\alpha2$ conformational epitopes from $\alpha3$ domain epitopes in this system. They observed little if any formation of $\alpha1\alpha2$ domain conformational epitope either in the absence of co-translated β2m or the presence of a minimal antigenic peptide and profound synergy of a nonamer peptide and β2m on the formation of the native conformation of $\alpha1\alpha2$ as well as $\alpha3$. A small but detectable number of molecules with the native $\alpha1\alpha2$ domain structure but with non-native $\alpha3$ structure were detected in sequential immunoprecipitation experiments, indicating that the folding of $\alpha3$ is not an absolute prerequisite for the formation of native $\alpha1\alpha2$. A result consistent with this has also been reported by Elliott et al (62) who observed the induction/stabilization of $\alpha1\alpha2$ conformational epitope in a mutant of H–2D^b in which the $\alpha3$ domain was deleted. Silver et al (184) and Parker et al (185) have shown that assembly of properly folded class I heavy chain–β2m complexes from unfolded heavy chain and β2m proteins requires specific peptide. These latter data, along with refolding studies on cell surface molecules (165) establish that intracellular chaperones are not essential to such assembly, although some data have been interpreted to indicate a critical role for the intracellular environment in efficient class I trimer formation (164, 186). These results have significant implications for our understanding of the capture of peptides by class I molecules within cells. The relationship of peptide structure to stability of the class I molecule also bears on the lifetime of exported class I and loading of class I with exogenous peptides.

QUANTITATIVE CHARACTERISTICS OF THE CLASS I/PEPTIDE/β2M INTERACTION A complete appreciation of the immunological role played by the synergistic interaction of the class I heavy chain with peptide and β2m requires a quantitative understanding of the contribution of each of these components not only to the formation of the complex, but also to its stability. Although direct evidence for the binding of purified class II molecules to labelled synthetic peptides was convincingly demonstrated as early as 1985 (10, 11), early attempts to measure similar kinds of binding in the class I system were unsuccessful. In retrospect, these difficulties can be seen to derive primarily from the use of synthetic peptides longer than the optimal lengths that are required for effective binding to class I, in part

from the high occupancy of mature class I molecules expressed by normal cells, and in part from the failure to provide adequate β2m during the binding phase. The identification of sources of class I molecules with a high proportion of functionally empty binding sites has permitted the beginning of quantitative evaluations of peptide/MHC class I interactions.

Christinck et al (187) measured an association rate constant of about 700 M^{-1} sec^{-1} at 37°C, and Boyd et al (188) found a somewhat slower one of about 10 M^{-1} sec^{-1} at 37°C. Both these values, measured on "mature" molecules that had already reached the cell surface or that had been secreted, are considerably lower than one might expect for a diffusion-limited association. This suggests either that the apparent on rates measured are low because they reflect the time-dependent generation of new empty sites, or that a slow but critical conformational change is required for peptide binding. More precise measurement of binding of peptide to known empty molecules will be required to better understand this issue.

Kinetic dissociation rates may be a better indication of the biologically relevant cell surface lifetime of MHC class I/peptide complexes. Cerundolo et al (178) found k_{off} values of nonamer peptides at 37°C corresponding to $t_{1/2}$ of about 3 to 7.4 hr, and Christinck et al (187) measured values for k_{off} at 37°C equivalent to a $t_{1/2}$ of about 30 min for a 17-mer. Using purified class I H-$2L^d$ protein and an iodinated nonamer, Boyd et al (188) observed a k_{off} corresponding to a $t_{1/2}$ of 1.9 hr when care was taken not to dilute the sample. The rates of loss of peptide or dissociation of bound β2m are similar, emphasizing the interdependence of peptide and β2m association with heavy chains. The general finding that longer peptides have a more rapid dissociation rate from class I than do shorter ones clearly reflects the biological importance of intracellular generation of optimal peptides for class I binding. In addition it raises the possibility that longer peptides bound to class I, then released relatively rapidly at the cell surface, give rise to empty sites that can be occupied by exogenous peptides.

Class II

RELATIONSHIPS BETWEEN PEPTIDE BINDING AND MHC CLASS II BIOCHEMISTRY

The demonstration that peptides made a critical contribution to assembly and maintenance of mature class I molecules raised the question of whether peptide played a similar role in class II assembly and stability. The first suggestion that MHC class II molecules might alter their structure upon peptide interaction came from analysis of the kinetics of peptide-class II association. The low ($\sim 2\ \mu M$) affinity constant first measured for class II-peptide binding (10) was soon shown to arise from the slow formation of long-lived peptide-MHC class II molecule complexes [slow-on/slow-off kinetics] (11). But why should the apparent association rate be so slow?

Sadegh-Nasseri & McConnell (189) demonstrated that fast, specific peptide–class II association did occur, with a $t_{1/2}$ of approximately 2 min. However, the complexes that rapidly formed also rapidly dissociated, with a $t_{1/2}$ of about 10 minutes. Long-lived complexes ($t_{1/2}$ of >30 hr) formed slowly under these same conditions. These investigators concluded that the slow step in producing long-lived complexes involved a slow or infrequent structural transition in the MHC class II–peptide complex that was necessary for attaining the stable binding state.

Additional evidence for changes in class II structure came from studies on the effects of denaturants on the SDS-PAGE properties of mouse class II molecules. Several groups had noted that isolated class II molecules remained associated as noncovalent dimers during SDS PAGE, if the samples were not heated before application to the gel (190–192). Dornmair et al (193) showed that heat or low pH led to a two-step loss of both bound peptide and this characteristic gel mobility, with mild conditions leading to a more slowly migrating dimer termed "floppy," and stronger denaturation resulting in molecules running as separate α and β chains. Work by Mellins et al (194) also suggested a relationship between class II structure and peptide binding. These investigators identified mutant cells with wild-type class II α and β chains that were markedly deficient in presenting intact protein antigens to T cell clones. These mutant cells also produced class II dimers that failed to stay together in SDS-PAGE. Mellins et al suggested that the loss of class II stability was related to the loss of antigen presenting capacity.

Direct evidence that peptide contributed to the characteristic stability of class II dimers came from Sadegh-Nasseri & Germain (35). Using the SDS-PAGE method to visualize changes in $E\alpha E\beta^k$ molecule structure, they confirmed the results of Dornmair et al (193) that removal of bound peptide by low pH was associated with a quantitative loss of dimer stability, and first demonstrated that reintroduction at low pH (195) of peptides known to bind to $E\alpha E\beta^k$ could restore the stable dimer structure in proportion to bound labelled peptide. Analysis of intracellular changes in class II structure after initial biosynthesis (196) connected the previous observations on mutant cell lines with these in vitro data on the role of peptide in class II stability. Newly synthesized class II molecules produced by mouse spleen cells were shown to be SDS-labile, the characteristic of peptide-free molecules. After 2–4 hours, a time span that corresponded to the known residence of class II molecules in post-medial Golgi compartments before reaching the cell surface, these molecules acquired the stable dimer structure indicating peptide acquisition. Similar observations were also made by Davidson et al (197). Addition of large amounts of free antigen to cultured spleen cells led to marked increases in the conversion

of newly synthesized molecules to stable dimers and up to a two-fold increase in surface class II expression. The majority of these additional stable dimers did not come from class II molecules that would have remained in the SDS-labile state; rather, they came from class II molecules that were otherwise destined for intracellular degradation. Pierce and colleagues (198) also noted increased total yields of MHC class II molecules from cells exposed to high concentrations of antigen. These results indicate that a substantial fraction of class II does not find usable peptides in the processing/loading compartment under normal conditions and may not reach the cell surface.

INTRACELLULAR EDITING OF EMPTY CLASS II MOLECULES AND THE LIMITATIONS OF THE SDS-PAGE ASSAY FOR CLASS II PEPTIDE BINDING The fate of class II molecules exposed to low pH in vitro with or without added peptide has shed some light on the physiological significance of this effect of antigen on class II expression (38). Exposure of newly synthesized $A\alpha^kA\beta^k$ complexes in detergent lysates to pH 5 at 37°C results in Ii dissociation and the inclusion of most of the $I\text{-}A^k$ in protein aggregates. If a good $I\text{-}A^k$ binding peptide such as HEL 46–61 is included during the low pH treatment, more of the class II is recovered in the soluble lysate, and this additional class II is in the stable dimer form. Thus, peptide binding seems to protect class II from entering into protein aggregates upon exposure to low pH. Stern & Wiley (199) have reported that peptide- and Ii-free class II molecules produced in a baculovirus system tend to aggregate, and that this aggregation is reversed by peptide binding. When these in vitro results are combined with the data on rescue of class II from degradation in vivo by antigen addition, it appears that there is an intracellular "editing" mechanism for reducing the movement of empty (peptide-free) class II molecules to the cell surface. Thus, class II, when freed of Ii in the late endocytic compartment, would face two possible fates—successful binding of peptide with an associated conformational change that protects the complex and allows its eventual egress to the cell membrane, or aggregation of empty dimers that ultimately leads to class II destruction. This mechanism would ensure a high representation of useful (i.e. peptide-containing) class II molecules on the cell surface. The data therefore support a role of peptide in regulating not only the structure of class II molecules in vitro, but more importantly, the fate of class II molecules within the cell, results analogous to the effect of peptide on class I.

Not all class II dimers that survive for extended periods of time in living cells show the SDS-stable phenotype, and a substantial cohort of surface membrane-expressed class II molecules of normal cells are unstable (196, 197). If most class II molecules reach the cell surface because of association

with peptide, this raises a question about the relationship between behavior in SDS-PAGE and peptide binding status. Lanzavecchia et al (200) have reported SDS-labile molecules that nevertheless appear to have bound peptide, and the life-span of these complexes on the cell membrane is similar to biochemically stable complexes, suggesting a tight peptide-MHC association despite the lack of the SDS denaturation-resistant phenotype. These results may indicate that the dimer state that resists SDS dissociation results from two distinct effects of peptide. One is a contribution to dimer stability by interaction of the peptide with portions of both the α and β chains. The second component appears to involve a change in the structure of the class II $\alpha\beta$ dimer per se that adds to the intrinsic stability of the class II molecule. This conformational stability presumably accounts for the strong association of α and β chains seen in the floppy form even after peptide release. This evidence for biologically significant peptide binding without entry into the fully stable state resistant to SDS denaturation reveals the limitations of this assay for peptide binding. As a general rule, class II molecules that have never bound peptide ["empty molecules"] dissociate in the SDS gel system (196, 197); dimers resistant to SDS dissociation are generated by and contain peptide ["loaded molecules": (35, 196–198, 201)]; however, dimers that come apart into α and β chains in such gels may nevertheless have contained peptide before dissociation in the SDS buffer (200). Thus, the method is very useful for establishing that peptide binding has occurred, based on the presence of stable dimers, but the absence of such dimers cannot prove a lack of peptide binding.

MECHANISTIC ASPECTS OF THE INTERACTION OF PEPTIDE AND MHC CLASS II MOLECULES These observations concerning the structural effects of class II-peptide interaction can be combined with data on the effects of pH on class II/peptide binding to suggest a model for the various steps that result in formation of long-lived peptide-MHC class II complexes.

Newly synthesized class II α and β chains associate with Ii and fold into an unstable dimer with an empty binding site (196, 197). Following removal of sufficient Ii to reveal the binding site, peptide associates with this unstable form of class II. However, this initial peptide–class II interaction does not result in efficient formation of long-lived complexes or stabilization of the class II molecule itself. Long-lived binding appears to require a conformational accommodation of the class II and peptide, and the likelihood that this change will occur during the short-lived interaction ($t_{1/2} < 10$ min) of peptide and class II that constitutes the initial low affinity state dictates how rapidly stable peptide-MHC class II complexes will accumulate. At neutral pH, most of the rapidly bound peptide dissociates from the class II molecule before the structural change(s) needed for stable

binding can occur, and in each cycle of binding and rapid release, only a very small number of the complexes formed make the transition to the slowly dissociating state. This gives rise to the slow accumulation of long-lived complexes observed by Sadegh-Nasseri & McConnell (189) following the initial rapid short-lived complex formation, and matches the binding rates seen in the work of most other investigators who do not look for the initial rapid phase.

Following the seminal study of Jensen (195), several investigators have shown that low pH (4.5–5.5) increases functional class II peptide presentation and total bound peptide assayed by methods detecting slowly dissociating complexes (35, 201–206). Part of the low pH effect is increased dissociation of previously bound peptide, making additional binding sites available (203, 206). However, the studies of Sadegh-Nasseri & Germain (35) first showed that low pH can also affect the rate of stable complex formation even when already unoccupied class II molecules free of Ii are analyzed, a result supported by the more recent extensive studies of Reay et al (206). Low pH may accelerate the generation of stable complexes by increasing the conformational accessibility of the class II molecule, thus increasing the likelihood of proper peptide-MHC structural alignment in any individual peptide–class II pair during each binding cycle. In some cases, peptide–class II binding site accommodation occurs without the usual accompaniment of stabilization of the dimer itself, giving rise to molecules with slow-peptide dissociation properties that are nevertheless not resistant to separation upon SDS-PAGE. These molecules are the converse of floppy dimers, which show strong dimer association without peptide-dependent stabilization of the conformation of the binding site.

Recent work of Jensen (207) suggests that at endosomal pH (5–5.5), peptide-class II conformational accommodation can take place efficiently and generate a complex resistant to the denaturing effects of the low pH, leading to long-lived peptide-class II association without the need for neutralization. This result indicates how peptide capture by class II molecules could be very effectively augmented in a low pH environment from which the complexes might not immediately exit (149).

All the observations contributing to this model of pH effect involve experiments with class II dimers either synthesized in the absence of Ii (199, 201, 206) or free of Ii and requiring denaturing conditions to remove previously bound ligand (35, 202, 204, 208). It therefore remains possible that the need for low pH reflects a requirement to remove the class II from a state of misfolding that occurs in the combined absence of peptide and Ii (209). Evidence for a conformational difference between class II dimers produced in the presence and absence of Ii exists (143, 210), and renaturation from dissociating conditions could easily result in a non-native

state. Class II dimers properly assembled in the presence of Ii would not need such refolding and might be very effective at peptide capture over a wide pH range after Ii removal. Given the likelihood that class II binding of peptide occurs in an acidic compartment, the optimal capture of peptide by class II at low pH seems physiologically relevant. However, the mechanistic details described above of how pH affects the rate of formation of stable peptide–class II complexes may pertain only to experimental preparations of class II molecules.

SITES OF ANTIGEN PROCESSING FOR AND PEPTIDE ACQUISITION BY CLASS I AND CLASS II MOLECULES

Class I

Because the NH_2-terminal peptide binding region of class I is not available in the cytoplasm, due to co-translational import of the heavy chain into the endoplasmic reticulum, the first possible site of peptide–class I association is the ER lumen. Studies with mutant cells provide data for early intracellular association of class I molecules with peptide. The presence of unstable class I heavy chain-β2m complexes in the ER of mutant cells such as RMA-S (34) and .174 × CEM.T2 (211) indicates a deficiency in peptide association and argues that the stable heavy chain-β2m complexes formed within 15 minutes of synthesis and present at a premedial Golgi site in normal cells result from peptide acquisition in this location. RMA-S cells also show a prolonged association of class I heavy chain β2m complexes with p88 (37), consistent with the view that release from this protein in the ER requires the stabilizing effect of peptide on the class I-β2m complexes.

Given this putative location for class I–peptide interaction, and the evidence that the primary source for peptides binding to class I is the pool of proteins normally residing within the cytoplasm, it is clear that either proteins considered to be exclusively cytoplasmic must at some low level enter the ER for degradation or that peptides generated directly from cytoplasmic proteins must cross a membrane to interact with the lumenal binding domain of the class I molecule. Two closely linked genes within the class II region of the MHC that have strong homology to the ABC family of transmembrane transporters have been identified (212–216). The accepted names for these genes are now TAP–1 and TAP–2 (transporter of antigenic peptides). Transfection studies into RMA-S (217, 218) and human cells with mutations involving the TAP genes (219, 220) have established that the class I assembly and antigen presentation defects in these cells can be corrected by establishing adequate expression of these

two TAP proteins, which form an ER membrane-associated heterodimer (221).

Human TAP proteins show allelic polymorphism (221) and studies in the rat have revealed significant functional polymorphism of at least the TAP–2 gene. Powis et al (222) have cloned, sequenced, and expressed the four allelic TAP–2 genes of the rat (previously called mtp2—212) encoded by the cim^a (for "class I modifier"), cim^b, cim^l, and cim^u genes (223), and have demonstrated that polymorphism in this gene results in a difference in the phenotype of the product of the class I gene RT1.A. This phenotype reflects variations in the spectrum of self-peptides associated with RT1.A molecules, which then affects the intracellular transport rate of this class I molecule as well as its recognition by antibodies and T cells.

Despite these data that all lead to the same model, namely that a heterodimer of TAP–1 and TAP–2 forms a peptide transporter that conveys cytoplasmic peptides into the lumen of the endoplasmic reticulum where they are available for coassembly with the newly synthesized class I heavy chain and β2m, it has not yet been directly established that TAP–1/TAP–2 acts as an active (or even passive) transporter of peptides generated in the cytosol. Uncertainty about the actual biochemical activity of the TAP heterodimer stems from the failure of several microsome-based in vitro assays to demonstrate TAP-dependent transport of peptides into the microsome lumen (224, 225). Peptide entry can be observed, but it does not seem to correlate with either the presence of TAP in the microsomes or require ATP.

There are no direct experiments addressing the mechanisms of peptide generation in the class I pathway. The best available evidence indicates that peptides are primarily generated in the cytosol, given the superior efficacy of signalless hemagglutinin protein, for example, to be presented by class I despite a markedly diminished or absent ability to enter the ER as an intact protein (226). Closely linked to the TAP genes in the MHC are another pair of novel genes [the LMP genes] that each encodes a protein with strong homologies to known components of a multicatalytic protease termed the *proteasome* (227–230). This molecule is a very large assembly of some 16–20 components that is conserved from bacteria to vertebrates, and it appears to be responsible for degradation of proteins in the cytosol as part of the general housekeeping functions of the cell (231). The LMP proteins have some homology to serine proteases, but no enzymatic activity has yet been demonstrated. The close linkage of the LMP genes with the TAP genes, their location in the MHC, and their association with a known proteolytic complex all suggest that LMP mediate either a specialized proteolytic function of proteasomes, for example, the production of peptides of the proper length for binding to class

I or for transport via the TAP heterodimer, or that they coordinate peptide generation by the proteasome with access to and transport via the TAP heterodimer for ultimate association with class I molecules in the ER lumen.

Class II

SITES OF CLASS II PEPTIDE BINDING AND THE ROLE OF Ii Do the peptides in the endoplasmic reticulum that can bind to class I molecules associate with class II and have any effect on $\alpha\beta$ dimer association and transport? TAP-deficient fibroblasts lacking Ii assemble and export class II molecules to the same extent as TAP-expressing fibroblasts (G. Otten, E. Bikoff, R. Germain, unpublished observations). This indicates that peptide does not play an essential role in the formation or transport rate of unstable class II dimers. The absence of stable class II dimers among the molecules produced in Ii-negative cells is consistent with a low efficiency of peptide binding in this location, but the possibility that the peptides available in the ER can only generate SDS-sensitive dimers cannot be ruled out at present.

The neutral pH of the ER may contribute to poor peptide acquisition by class II in this location, but under the normal physiological conditions another factor actively prevents class II peptide loading in this location. Roche & Cresswell (232) were the first to provide evidence that the association of Ii with class II inhibited stable peptide–class II binding. These data on extracellular peptide binding by class II were confirmed by Teyton et al (233) and by Roche et al (136). Evidence for Ii inhibition of peptide acquisition by class II molecules during biosynthesis and transport within living cells was provided by Germain & Hendrix (196) and Davidson et al (197), who used the SDS-PAGE stability assay to show that class II associated with intact Ii lacked evidence of peptide binding.

The mechanism of Ii interference with class II peptide binding is not well-characterized. A direct block of the peptide binding groove is consistent with the ability of a soluble form of Ii to inhibit monoclonal antibody binding to the polymorphic domain (233). Alternatively, Ii may inhibit the structural transition(s) needed for stable binding of peptide (196, 232). The finding that leupeptin prevents the generation of SDS denaturation resistant class II dimers in vivo (149; R. Germain, C. Layet, unpublished observations) could indicate that the fragment of Ii remaining associated with class II under these conditions prevents effective peptide binding by a mechanism other than direct site blockade.

Because Ii prevents stable peptide–class II association, intracellular capture of peptide by class II molecules must take place after the inhibitory portion of Ii has been removed. This makes it likely that much if not all

normal peptide capture occurs in later parts of the endocytic pathway, because little cleavage of intact Ii has been observed in early endosomes (130, 148). Unfortunately, no experiments have addressed whether peptide binding is affected by the association of class II with the more NH_2-terminal portions of Ii that remain when cathepsin B cleavage is inhibited. Therefore, class II molecules associated with such fragments in the late endosomes/prelysosomes that constitute the primary site of class II accumulation may not actually be available for effective peptide binding. Class II might arrest there due to a delay in removing the last fragment of Ii, following which the class II could move rapidly through an even later portion of the endocytic/lysosomal pathway, acquiring peptide during this transit. Such a scenario would agree with the very low amount of class II in lysosomes (126, 127) and yet would fit results suggesting that processing of proteins is most efficient in these organelles (150, 234). Alternatively, peptides could load after partial Ii removal, and following further dissociation of Ii, perhaps itself promoted by the conformational changes that accompany class II binding of peptide, the loaded class II molecules could exit the cell directly from this location. If so, then either lysosomally generated peptides are not a source of antigen for class II binding, or a shuttle mechanism must exist for moving these materials to the site of accumulated class II (150). Interestingly, Dice and colleagues have reported the release from lysosomes of peptide fragments appropriately sized to bind to class II molecules (235). In either case, the observation by Neefjes & Ploegh (149) that stable class II dimers are generated at an intracellular site up to several hours prior to their appearance on the cell surface indicates that once loaded with peptide, either release from remaining Ii fragments or passage to the cell surface from the site of loading can be a slow event.

Attempts to relate Ii expression with functional class II presentation of exogenous antigens have led to conflicting results. Stockinger et al (236) reported that co-expression with class II of the p31, p41, or both forms of Ii had a potent enhancing effect on presentation of some soluble protein antigens. However, different results were obtained by Peterson & Miller (147), who found no enhancement of ovalbumin presentation by Iip31, but who did note a decrease in the capacity of the cells to present peptide, which suggested that the proportion of occupied class II on the surface was increased by Ii expression. These same investigators have found that the p4l form of mouse Ii could augment presentation of ovalbumin and other antigens to some T cells, whereas the p3l form lacked this ability, and neither form improved presentation of the same proteins to other T cells (237). Nadimi et al (238) noted an increase in presentation of hen egg lysozyme but no increase in presentation of ribonuclease by cells co-

expressing class II and Ii, in comparison to transfected fibroblasts with only class II expression. These variable results may indicate that Ii is not essential for class II presentation, but that it does increase presentation of some specific peptides, perhaps by affecting the localization of class II within subcompartments of the endocytic pathway or the likelihood of antigen–class II association in individual endocytic vesicles.

STUDIES WITH DRUGS AND INHIBITORS Modification of the cellular environment using drugs or temperature shifts has also been used to probe the class II processing and presentation pathway. Agents that raise intracellular pH, such as chloroquine or NH_4Cl consistently interfere with presentation of antigen by MHC class II molecules, unless preformed peptides are used to load surface class II molecules (239). Although a decrease in the generation of peptides suitable for binding to MHC class II remains a likely reason for the effects of these agents, the published experiments have all been performed using cells co-expressing Ii. Because acid proteases are necessary for the removal of Ii from the class II-Ii complex (121, 124, 240, 241), and such removal is necessary for both exposure of a functional class II binding site and for release from intracellular retention, assays using T cells for detection of surface membrane class II–peptide complexes cannot determine whether a lack of peptide or a failure of loading / transport or both is (are) the explanation for the inhibitory effects of these drugs. A similar argument applies to the inhibitory effects of leupeptin. Protein synthesis inhibitors and the drug brefeldin A have given contradictory effects on class II–dependent presentation in different laboratories (242–249) and have not proved especially useful in identifying the steps in class II presentation.

LOADING OF PROCESSED ANTIGEN ON RECYCLING OR SURFACE CLASS II MOLECULES Significant attention has been given to the possible role of class II recycling and reloading in antigen presentation. Some studies have found only a low rate of internalization of class II molecules (125, 250, 251), but others report substantial internalization and even recycling (154, 243, 252, 253). Such variable results make it difficult to determine the extent to which this route contributes to class II antigen presentation. The pH reached in the early endosomal sorting compartment is not low enough to displace tightly bound peptides, at least in in vitro analyses (202, 206), making it unlikely that the rapid rate of extinction of functional presentation of such peptides results from their removal and replacement due to recycling based on pH stripping alone (243, 254). Marsh et al (255) have reported a pH-dependent loss of peptide–class II association in living cells, but these studies do not address reuse of the class II molecules, as opposed to pH-dependent proteolysis as the ultimate fate of these molecules.

INTRACELLULAR CHAPERONES FOR PEPTIDE TRANSPORT AND LOADING OF CLASS II Pierce and coworkers have described a pair of molecules termed p72/74 (256) that are members of the hsp70 family (257) and that show the unusual property of being expressed on the cell membrane of B cells (258). Because molecules in the hsc 70 family, including p72/74, have been shown to possess peptide binding capacity (259), Pierce and colleagues (260) have suggested that such molecules might scavenge peptide or partially degraded proteins, preventing terminal degradation in the very proteolytically active environment of prelysosomes/lysosomes (234). They might constitute the shuttle mechanism for provision of such processed antigen to class II accumulated in earlier endocytic compartments (150, 234). It is intriguing that two groups have provided some evidence for structural homology between the peptide binding regions of hsp70 molecules and MHC molecules, perhaps indicating an evolutionary relationship between the two families of peptide-binding proteins (261, 262).

A defect in expression or trafficking of these hsc 70 family members or some other peptide-binding shuttle protein encoded near the class II region of the MHC could give rise to the defective exogenous antigen presentation seen in the mutant cells described by Mellins et al (194) and by Riberdy & Cresswell (263), by precluding the delivery of processed antigen generated in lysosomes to available class II binding sites in late endosomes/prelysosomes. The suggestion that the defect in presentation of exogenous antigens by cells with mutations in non-class II genes within the MHC lies in bringing peptides and class II together is consistent with the demonstration that these cells do have some peptide stably associated with their class II molecules, much of which derives from invariant chain (P. Cresswell, personal communication). Such Ii peptides would be among the few that would be available at high concentration in a cell that lacks a mechanism to retrieve other peptides from lysosomes in which a reducing environment and highly active proteases could efficiently degrade compact globular proteins (150). At least superficially, the characteristics of these mutants affecting the class II pathway and the TAP mutants with defective class I antigen presentation are similar (55, 264). In each case, the defect would lie in a transport mechanism, rather than in either peptide–MHC molecule loading per se or in generation of processed antigen. The mutations in both cases overlap in the genome (215, 265), consistent with a cluster of peptide transport genes in this region of the MHC. As attractive as this model is, it must be emphasized that there is little evidence supporting the existence of such a transport machinery in the class II pathway, and defects in production of peptides from antigen, in antigen uptake itself, or other aspects of the class II processing pathway could be the locus of the defect in the mutant cell lines. Identification of the affected gene(s)

in these cells will clearly aid greatly in advancing our understanding of how the class II system works.

THE ENIGMA OF "ENDOGENOUS" ANTIGEN PRESENTATION BY CLASS II MOLECULES If little or no peptide association with class II takes place in the ER due to pH and Ii effects, then how can the numerous observations of class II presentation of peptides derived from so-called "endogenous" proteins (247, 266–274) be explained? It is useful to divide the proteins studied in such experiments into three groups: one that includes proteins known to reach the cell surface and hence to be available for endocytic entry; one that includes proteins apparently retained within the ER; and one including cytoplasmic proteins. Proteins in the first group are operationally "exogenous" and clearly can be processed after they reenter the cell by the standard endocytic route. The high efficiency of this pathway for membrane anchored molecules or other proteins able to bind to such molecules is expected, based on the 10^2–10^4-fold increases in antigen presentation seen using ligands binding to surface immunoglobulin (275, 276) or cross-linked to surface proteins with substantial rates of entry into endosomes (277, 278). In some experiments, putatively soluble forms of endogenously synthesized antigen have been examined and found to be efficiently presented (271, 272, 274). Cell mixing experiments showed little or no cross-presentation. However, such experiments do not take into account the very high local concentration of protein present in the immediate vicinity of the actual secretory cell itself, which would facilitate antigen uptake, the possible association of a fraction of the otherwise soluble protein product with membrane components of the producing cell that would result in rapid recycling into the endocytic system (a route unavailable to admixed cells), or the possible direct intracellular routing of a fraction of the soluble protein from Golgi to endosomes, perhaps as part of the quality control mechanisms that might capture and route misfolded proteins to degradative compartments. Thus, for the present time, such experiments cannot be taken as firm evidence for a distinct pathway of processing and presentation via class II molecules, although this remains a possibility.

The second category of proteins, those imported into, then retained in the endoplasmic reticulum [for example, by the presence of an added KDEL signal] have been used to argue strongly for a non-endosomal site of antigen processing and peptide binding to class II molecules. Among the several reports using this experimental strategy, one of the most complete is that of Weiss & Bogen (279). Because synthesis of a leaderless version of the immunoglobulin light chain antigen examined in this study did not lead to T cell stimulation, the authors concluded that processing and

peptide binding was occurring in the ER. When one examines the quantitative aspects of this study, however, a different conclusion seems possible. The mutant chains, despite being rapidly and efficiently degraded, were markedly less effective in creating a stimulatory presenting cell than the wild type chain that largely resists such proteolysis. Thus, the situations generating more peptides gave less presentation, in some cases by several orders of magnitude, a result at odds with the ER being the relevant site of processing and peptide association with class II. Second, within the limits of the dose-responses performed, those constructs with the greatest "leak" out of the ER were the most potent [in fact, the wild-type chain that was most efficiently secreted was the best antigen], and those that were best retained were least stimulatory. It therefore seems likely that the relevant cohort of light chain protein was not that major fraction followed biochemically and shown to reside in the ER, but rather that small fraction that escaped this compartment, possibly moving to the endocytic pathway, where degradation and peptide loading on class II took place. Again, further study is needed to determine if presentation of such antigens occurs by a novel route.

The third group of antigens is the most intriguing. Jacobsen et al (268) first reported on the ability of a cytoplasmic antigen, the measles virus matrix protein, to be presented by MHC class II molecules. Subsequent work by a number of laboratories has documented class II antigen presentation of what are considered to be strictly cytoplasmic proteins (273, 280). Mixing experiments ruled out trivial explanations such as bulk release and uptake from the medium into the endocytic pathway. How can these data be reconciled with the general model of class II processing and presentation? No evidence is presently available on the actual site of peptide–class II association under these conditions. Chloroquine can inhibit presentation in at least one study of such a cytoplasmic antigen (280), raising the possibility that some antigen or peptide from the antigen has moved to the endosomal system for association with class II. In this case, the question becomes not where the peptide loads—this would be the same as for all other known ligands of class II molecules. Rather, it would be the site of peptide generation and the pathway followed by antigen or peptide to reach the endosomal compartment. This emphasizes that the class-specific function of MHC molecules relates primarily to their preferred site of peptide loading, which only indirectly and incompletely dictates the protein sources (exogenous/endogenous) of such peptides.

Hsc 70 molecules participate in moving cytoplasmic proteins or peptides into lysosomes (281), and they provide one possible route for moving such cytoplasmic antigens into the conventional class II binding environment (274). Another possibility is autophagy (282), which involves the move-

ment of cytoplasmic components into the degradative system by vesicle formation. A more esoteric route is for peptides to be generated by the standard class I pathway and move into the ER via the TAP route presumably used by class I ligands. Such peptides could bind to class I in the ER; when this class I moved into the endocytic pathway for degradation, peptide could be released and captured by class II. This might be one explanation for the TAP dependence of presentation of a cytoplasmic minigene product that ultimately is found associated with class II and whose presentation, in contrast to class I associated peptides, is chloroquine-dependent (280). Finally, we do not yet know if Ii prevents all peptide–class II association or only inhibits slow-off rate binding. Peptides made in the cytosol and then imported into the ER thus might weakly associate with class II-Ii complexes in the ER and be transported with these complexes to the endocytic compartment where they would dissociate, then bind to these or other class II dimers under the acidic conditions that promote rapid formation of highly stable complexes. This would again explain the chloroquine-dependence of this event.

CONCLUSION

Although many details, and possibly even major elements of the antigen processing and presentation pathways remain to be identified and described, a basic picture of the biochemical and cell biological events involved in MHC molecule synthesis, transport, peptide acquisition, and surface expression has begun to emerge. A consistent underlying theme is the importance of protein folding events and compartment-specific chaperones in the class-associated functions of MHC molecules.

Misfolded or incompletely assembled proteins, if present in the ER, are usually retained there by association with stress-related proteins or molecular chaperones that are believed to participate in preventing irreversible aggregation of such molecules and in giving them an opportunity to attempt to reach a properly folded/assembled state (283). Much of what we know about the intracellular fate of MHC molecules can be readily explained if we imagine that the polypeptide chains encoded by MHC genes are functionally equivalent to protein deletion mutants. Such mutants lack the necessary information in their own primary sequence for entering a conformational state that these quality control mechanisms of the cell perceive as properly folded. For class I molecules, no specialized chaperone equivalent to Ii is available to hide the peptide patches that are recognized by the ER resident chaperones, and retention occurs until peptide has stabilized a properly folded structure that eliminates exposure of these sites. This event involves increasing the affinity of the heavy chain for β2m

(159, 165, 178, 188). Too great an affinity between empty class I heavy chains and β2m would result in export of unoccupied class I molecules and inefficient endogenous peptide presentation; a requirement for too great a contribution of peptide to the heavy chain-β2m interaction would make the number of useful peptides too small. Thus, heavy chain-β2m affinity appears to have evolved such that in the absence of peptide there is a small proportion of heavy chains showing transient proper folding in association with β2m. This would promote escape from p88 association and egress from the ER (37). Some chaperones move through the entire set of Golgi stacks (284) and may recapture class I upon dissociation of the loose heavy chain-β2m complexes in these late secretory locations (285). Because the adaptation of the heavy chains and β2m involves intra-species selection, trans-species heavy chain-β2 combinations that have continued to evolve apart from each other might well have too great an affinity in the peptide-free state, allowing significant transport without peptide capture. This may account for the export of most mouse class I heavy chains from TAP deficient cells in the presence of human β2m (286).

Most class I will only exit the ER after capturing peptide and becoming stably associated with β2m. Peptides that only weakly stabilize this trimer form will contribute little to overall class I expression, as they will be replaced by better binders prior to class I expression due to equilibrium exchange, or they will result in molecules that fail to complete their trip to the cell surface. Nevertheless, the most poorly stabilized molecules that do reach the surface may be the primary source of free class I binding sites that allow exogenous peptide addition to sensitize a target cell (165, 180, 287). The remaining molecules will associate with optimal peptides. Once occupied by such peptides, the class I molecule is very stable (168, 178, 188). Because transport depends on this effect of peptide, surface class I expression is selective for peptide-bound molecules, reducing the frequency of empty class I molecules that might interfere with recognition of useful peptide-class I complexes.

Thus, class I structure and its requirements for attaining a state that avoids retention by intracellular chaperones dictates its acquisition of peptides in the ER. Few binding sites are free for capturing peptides in other locations, due to the tight association of the ER peptides with the class I molecules transiting to the cell surface. Because many potential antigens of pathogens such as viruses would be cytoplasmic proteins, because for viruses fusing directly with the cell membrane no direct deposition of protein would occur in the endocytic pathway, and because the first proteins produced by a virus during infection are typically cytoplasmic or nuclear proteins regulating viral replication, a mechanism is required for supplying peptides derived from such proteins to the class I binding

site in a topologically distinct compartment. This appears to have involved subverting the function of a preexisting protease system (228, 231), so that peptides (perhaps of a particular type suitable for the class I binding site—222) were made and delivered to a transport apparatus for movement across the ER membrane (the TAP system) (212–217, 219). In this sense, the generic structure of class I dictates where it gets peptide, and additional, closely linked gene products assure an appropriate supply of peptides in that location.

The same principles, but with important differences in the details, apply to class II (36). To focus class II on peptides outside of the ER, it was necessary to evolve a structure that was not dependent on peptide for its assembly and export, yet was poised for such peptide binding in another location. This requirement was met by the different domain organization of class II and especially the presence of two transmembrane regions in its component polypeptides (110). This allows a more stable assembly early after synthesis that is not dependent on peptide. Ii both keeps the class II binding site from being occupied with peptides before export from the ER (196, 232, 233) and also regulates the export process (141–143), but this multimer then needs a mechanism to access peptides derived from proteins outside the cell. This is accomplished by moving class II to the endosomal pathway (129–131). Because virtually all class II leaving the ER is associated with Ii (120, 122, 196), it is reasonable that the signals for such intracellular movement be in this latter molecule (129–131, 135). This allows subsequent dissociation of Ii to release class II for movement to the cell surface, without requiring removal of a portion of the class II itself to mediate escape from the endosomal pathway.

As for class I, the misfolded/readily denatured property of empty class II molecules is used by class II for preventing movement of unoccupied molecules to the cell membrane (38). For class II, peptide binding also leads to generation of a protein acting like a conventionally folded globular protein without a tendency to aggregate (38, 199) or be captured by any proofreading molecules/chaperones that would select a denatured protein for movement into the terminal degradation compartment. Also as for class I, this discrimination occurs at the site of peptide loading. Thus a common general mechanism, with important differences in the details, applies to both classes of MHC molecule. Class II interaction with peptide occurs efficiently in and may be optimized for the low pH environment in which it is meant to capture ligand (35, 195, 201–206, 208), and we see the results of this evolution in its weak capacity even when empty to acquire stably associated peptide at neutral pH.

Thus, the overall biochemical behavior of MHC molecules varies according to class independently of allelic polymorphism. Such behavior

strongly supports the view that the class I and class II families have evolved for different purposes defined by the intracellular environments in which they are optimized for peptide capture (26–29). Much of the auxiliary machinery supporting the two processing pathways has been identified, and the general outlines of each pathway discerned. The challenge now is to complete the cast of characters and to further define their specific modes of operation, especially the enzyme systems generating the peptides, the transport molecules and any selectivity they have for peptides, chaperones increasing the efficiency of loading, carrier proteins guiding transport of class II within the cell, and the route of class II movement from the site of loading to the cell surface. The next review of this topic in these pages will probably contain answers to many of these questions, and perhaps some surprises as well.

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REGULATION OF LYMPHOCYTE FUNCTION BY PROTEIN PHOSPHORYLATION

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Abstract

Variations in protein phosphorylation provide the predominant means of enzymatic regulation now known in biological systems, especially in the regulation of signal transduction from cell surface receptors. Analysis of these signaling pathways has proceeded especially rapidly in lymphocytes, in part because these cells can be isolated with relative ease and can in many cases be maintained in vitro for prolonged periods as clonal populations. During the past few years, both biochemical and genetic evidence has been adduced indicating that the antigen receptors of T and B lymphocytes associate functionally with nonreceptor protein tyrosine kinases. Similar data implicate protein tyrosine kinases in signaling from the CD4 and CD8 coreceptors and the β chain of the IL–2 receptor. Protein serine/threonine kinases and several different phosphatases also participate in the intracellular propagation of antigen receptor–derived signals. Here we review the lymphocyte surface receptors that are believed to act by altering protein phosphorylation, the kinases and phosphatases that are believed to regulate signal transduction in lymphocytes, and the implications of these results for the broader study of cell signaling mechanisms.

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INTRODUCTION

Lymphocyte surface receptors transmit signals to the cell interior that regulate proliferation, cytokine release, and a host of metabolic parameters. Broadly speaking, these receptors may be grouped into three categories. First, adhesion molecules influence migration, stabilize interactions with antigen presenting cells, and provide "handshake" signals that modulate activation responses (1). Second, receptors for cytokines interact with soluble or cell-bound factors, thereby transmitting signals that promote differentiation or proliferation. Finally, antigen receptors (the T cell antigen receptor and B cell surface immunoglobulin) associate with multimeric signaling complexes to permit rapid activation following encounters with non-self macromolecules.

Elucidation of the structures of these various receptor molecules has encouraged attempts to dissect the mechanisms whereby signals impinging on the lymphocyte surface are decoded within the cell body. Not surprisingly, signaling mechanisms previously defined in other systems contribute substantially to the regulation of lymphocyte function. However, lymphocytes offer certain advantages for those interested in broader aspects of cell signaling in that normal lymphoid cells can be isolated in large quantities in relatively pure form and can be propagated in a more-or-less synchronous fashion in vitro using well-characterized growth factors. Hence, an increasing number of researchers are directing their energies toward dissecting the physiology of lymphocyte responses. The hope that manipulation of these signaling systems can have beneficial clinical consequences has further energized this field of inquiry.

Even a cursory survey of literature in this active area reveals that alterations in protein phosphorylation contribute substantially to the molecular circuitry underlying lymphocyte signaling. There is little surprise in this because protein phosphorylation is the most common mechanism of regulation of enzymatic function known (2). Considerable effort has been expended in pursuing protein tyrosine phosphorylation in particular. This prejudice reflects in part the received wisdom (derived from study of growth factor receptors and the transforming products of retroviral oncogenes) that protein tyrosine phosphorylation, though representing a tiny fraction (usually less than 1%) of the total phosphorylation events observed in cells at steady-state, nevertheless defines a set of key regulatory processes affecting growth and differentiation (3). The bulk of protein phosphorylation occurs at serine and threonine residues and is mediated by kinases capable of interacting productively with substrates containing either acceptor residue (2). Phosphorylation of additional residues, notably

histidine, may ultimately prove to be important in lymphocytes, as has been shown in some bacterial systems (4). Events of this type have not yet been characterized, however.

Four categories of evidence permit inferences regarding the involvement of protein phosphorylation in lymphocyte signaling pathways. First, interaction of a receptor with its ligand may alter protein phosphorylation in a general way. This type of evidence emerges most frequently in the study of protein tyrosine phosphorylation, because highly specific antibodies can be used to identify phosphotyrosine (5), and because relatively few proteins in stimulated or resting lymphocytes contain phosphotyrosine residues at sufficient stoichiometry to permit detection. Biochemical approaches in many cases permit the demonstration of physical association between protein kinases and cell surface receptors when cells are gently disrupted using mild detergents. This provides a second category of evidence favoring the involvement of protein phosphorylation in a cell signaling pathway, especially where it can be demonstrated (as has been done in some cases) that ligand occupancy of the receptor structure stimulates the in vitro activity of the kinase. Two other types of experiment seek to implicate protein phosphorylation in lymphocyte signaling by manipulating the activity of protein kinases or phosphatases in whole cells. Experiments performed using protein kinase inhibitors, which suffer to some extent from the incomplete and ill-defined specificity of these reagents, frequently permit somewhat selective blockade of a lymphocyte signaling pathway. They provide a third category of evidence supporting the importance of protein phosphorylation as a primary regulatory event. Finally, recent advances in molecular genetics permit direct manipulation of protein kinase and phosphatase expression. In particular, lymphocyte-specific transcriptional regulatory elements have been used to generate transgenic mice with altered kinase repertoires, and gene targeting strategies have yielded mice with heritable defects in kinase expression. Such strategies promise to provide information regarding the importance of protein phosphorylation pathways in multiple lymphocyte subpopulations and throughout the maturational process that characterizes lymphocyte development.

In the following pages we inventory the protein kinases and phosphatases for which evidence supporting a role in lymphocyte signaling exists. We then consider the cell surface receptors that stimulate changes in protein phosphorylation, and we attempt to correlate these, where possible, with one or more of the known kinases. Our goal in assembling this material has been to adduce general lessons that will assist in formulating a comprehensive assessment of lymphocyte control mechanisms.

To this end, we present a schematic model of the layers of regulatory elements in the lymphocyte activation circuitry. While this process is often referred to as a regulatory "cascade," making deliberate reference to a paradigm derived from the study of glycogenolysis, it is probably more accurate to view phosphorylation as one of a set of regulatory mechanisms, including allostery, second messenger molecules, and posttranslational modifications generally, that together influence the way in which ligand binding to a lymphocyte surface receptor is perceived within the cell. As discussed below, considerable evidence suggests that protein tyrosine kinases exercise pivotal control of signaling from many lymphocyte receptor structures simultaneously.

PROTEIN TYROSINE KINASES IN LYMPHOCYTES

That protein tyrosine phosphorylation may underlie the early changes in lymphocyte behavior following stimulation with antigen or lymphokines has long been suspected, and several groups have demonstrated augmented accumulation of phosphotyrosine containing proteins resulting from these stimuli. Moreover, broad-spectrum protein tyrosine kinase inhibitors such as genistein (6) and the tyrphostins (7, 8) block most of the early changes in lymphocyte metabolism that accompany antigen receptor occupancy (see below). Kinases capable of phosphorylating tyrosine residues in substrate proteins form an elite group with many shared structural and functional properties (3). Included in this category is a set of membrane-spanning growth factor receptors (e.g. the receptors for platelet-derived growth factor and epidermal growth factor), as well as the products of cellular homologues of retroviral transforming genes (e.g. the products of the c-*src* and c-*fgr* genes). Convention dictates that the receptor-type protein tyrosine kinases be considered separately from those that do not contain a covalently linked extracellular component, though this distinction may ultimately prove irrelevant from the perspective of the architecture of signaling pathways (see below).

Growth Factor Receptors

Like most cells, lymphocytes express insulin receptors that are transmembrane protein tyrosine kinases. Although these receptors almost certainly influence intermediary metabolism, there is no persuasive evidence that insulin behaves as a primary or even ancillary regulator of lymphocyte activation or development. However, two other growth factor receptor-like protein tyrosine kinases, the products of the c-*kit* and *ltk* genes, may prove to be important control elements.

c-kit

The c-*kit* gene encodes a typical transmembrane receptor whose ligand has been identified as the product of the steel (*Sl*) locus. In mice, mutations at either *Sl* or c-*kit* (the latter called W mutants) yield comparable phenotypes, with defects in melanocyte, germ cell, and hematopoietic lineages (9). Analogous human c-*kit* mutations producing piebaldism have recently been described 3(10–12). In vitro co-cultivation experiments and transplantation studies performed in the mouse reveal that the defect in c-*kit* mutants is intrinsic to the hematopoietic stem cell; *Sl* mutant animals produce normal hematopoietic stem cells, but these fail to mature because of defects in the stem cell microenvironment (13). Steel factor itself (also known as mast cell growth factor, *kit* ligand, or stem cell factor) supports the production in vitro of progenitor cells from primitive precursor populations (14). Additional data adduced using bone marrow culture systems suggest that the steel factor/c-*kit* receptor pathway assists in regulating early antigen-independent B lymphocyte development (15). Specific targets of phosphorylation by the c-*kit*-encoded receptor have not been elucidated, and the pathways to which it contributes are unknown. Nevertheless, it appears that phosphorylation by this molecule influences differentiation of immature cells, but not activation of adult lymphocytes.

ltk

The *ltk* gene encodes a novel receptor-type kinase, identified in lymphocytes, leukemic cells, and neurons, which has a very short extracellular domain and an intracellular domain related to that of the insulin receptor (16–19). No ligand for the *ltk* product has been identified; indeed the protein may reside in the endoplasmic reticulum, where its activity may be controlled by alterations in redox potential (20). Hence this protein tyrosine kinase may participate in signal transduction pathways at a point downstream of conventional cell surface receptors (discussed in 20). Neither natural nor engineered mutations of the *ltk* gene have been identified.

Nonreceptor Protein Tyrosine Kinases

This group of kinases includes several critical signaling molecules that regulate both lymphocyte development and antigen responses, notably the *lck*- and *fyn*-encoded *src*-like kinases. The *src*-family of protein tyrosine kinases includes eight well-characterized members which, though distinctive, share common structural features (21–23). They range in size from 499 to 543 amino acids in length, and each contains a consensus myristylation sequence at the amino terminus, which promotes their interaction with

membranes. Within each kinase molecule, four "domains" can be defined based on comparison with other family members. The amino-terminal 70 residues constitute the first domain, which differs dramatically among the *src*-like kinases. Thereafter, two short "*src*-homology" intervals, the SH3 and SH2 domains, can be recognized. The SH3 domain is also observed in some actin-binding proteins, whereas the SH2 domain permits binding of phosphotyrosine-containing proteins (see 24 for review). The remaining half of the molecule represents a typical protein tyrosine kinase structure with recognizable motifs that assist in ATP binding, and a conspicuous tyrosine autophosphorylation site. Near the carboxy terminus, a second tyrosine phosphorylation site, believed to be the target of a distinct kinase, subserves a regulatory function. Mutations at this site that block phosphorylation typically result in enhanced kinase activity. In contrast, mutations at the centrally placed tyrosine autophosphorylation site exert less dramatic effects. Figure 1 presents a schematic diagram of $p56^{lck}$, which demonstrates these basic structural features (see 21 and 22 for a review of the *src*-like protein tyrosine kinases). Among the members of this family, the products of the *blk*, *fyn*, *lck*, *lyn*, and *yes* genes have been proposed to mediate signaling functions in lymphocytes (21, 22, 25).

$p55^{blk}$

Expression of the *blk* gene is restricted to B cells and B cell lines (23, 26). Transcription may be developmentally regulated since it is found in all pro-B, pre-B, and mature B cell lines examined, but not in plasma cell lines (27). The protein product, $p55^{blk}$, has also been found in association with

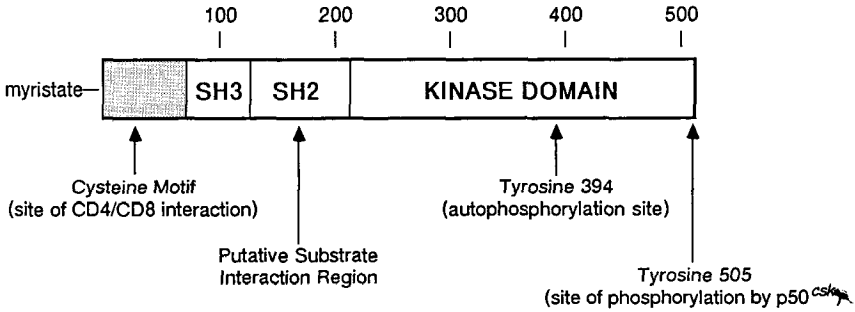

Figure 1 The structure of $p56^{lck}$, a typical nonreceptor protein tyrosine kinase. Shown is a cartoon outlining key structural motifs discussed in the text. The scale denotes distance (in residues) from the N-terminus. All src-family kinases share in common very similar SH3, SH2 and kinase domains but differ in their amino-terminal domains (shaded region) which probably assist in mediating interactions with cell surface receptors.

surface immunoglobulin (Ig) and reportedly is activated upon cross-linking of Ig (28). Activation of $p55^{blk}$ may parallel the overall increase in total phosphotyrosine observed after stimulation (28).

$p59^{fyn}$

The product of the *fyn* gene is expressed in neuronal and hematopoietic cells but is especially abundant in retinal and cerebellar neurons and in mature thymocytes (21, 23). Two alternative isoforms, the products of mutually exclusive splicing of alternative seventh exons, exist in mouse (29) and in human (unpublished data). The two forms, $p59^{fynB}$ (for the neuronal type) and $p59^{fynT}$ (for the hematopoietic cell type), differ within a 50-residue segment at the junction between the SH2 and kinase domains. Functional differences between the isoforms are still under investigation (see below).

In T lymphocytes, $p59^{fynT}$ appears relatively late in thymocyte development, and its expression correlates roughly with the ability of thymocytes to respond to T cell receptor (TCR)-mediated signals (30). This correlation led to early speculation that $p59^{fynT}$ might be involved in propagating signals through the TCR. Three observations strongly supported this view. First, TCR immunoprecipitates were found to contain low levels of $p59^{fyn}$ protein (31). Second, cocapping of $p59^{fyn}$ with the TCR/CD3 complex could be demonstrated under some circumstances (32). Hence there was good reason to believe that some fraction of $p59^{fynT}$ associated with the TCR complex. Finally, overexpression of $p59^{fynT}$ in a T hybridoma cell line (33), or in the thymocytes of transgenic mice (30), resulted in dramatic improvement in signal transduction from the TCR/CD3 complex. Despite this enhanced signaling capacity, thymocytes in the transgenic animals developed normally and appeared to undergo positive and negative selection normally (30, 34). In the same study, overexpression of a catalytically inactive form of $p59^{fyn}$ attenuated the proliferative response to anti-CD3 antibodies or lectin mitogens, once again without severely compromising T cell development (30).

These studies provide strong evidence favoring the view that $p59^{fyn}$ can impinge upon a signaling pathway leading from the TCR/CD3 complex to the cell interior. More recently, targeted disruption of either exon 7B (which contributes to the synthesis of $p59^{fynT}$) or exon 2 (which contributes to both *fyn* isoforms) of the mouse *fyn* gene has been reported (35, 36). In both cases, $p59^{fyn}$ was no longer detectable in T-lineage cells, and T cell signaling was significantly perturbed. However, the disturbance in signal transduction was incomplete. For example, although mature $CD3^+4^+8^-$ thymocytes in fyn^{null} animals were virtually nonresponsive to mitogens, superantigens, or allogeneic cells, $CD3^+4^+8^-$ peripheral T cells in these

mice exhibited only the modest deficits in signaling. Hence, while p59^{fyn} contributes to signaling from the TCR/CD3 complex, other mechanisms can clearly substitute effectively (35, 36).

Two aspects of these studies deserve special emphasis. First, although the responsiveness of mature thymocytes to TCR stimulation was almost completely abrogated, no gross defects in thymocyte development occurred. This result probably reflects the fact that those thymocytes most susceptible to selective influences normally do not express p59^{fyn} (30). Hence, although a more complete evaluation of T cell repertoires in *fyn*-deficient mice must be performed, it appears that the signaling pathways that direct positive and negative selection of thymocytes do not require expression of p59^{fyn}. A second intriguing finding was the extraordinarily selective expression pattern of the *fyn* isoforms. That is, although satisfactory expression of p59^{fyn}B was observed in the brains of mice bearing an exon 7B disruption, p59^{fyn} protein was not observed in thymocytes or splenocytes (35). This observation emphasizes that expression of individual *src*-family kinases is frequently restricted to specialized populations of hematopoietic cells (21; see below). Intriguingly, infection of an insulin-specific T hybridoma cell line with retroviruses encoding p59$^{fyn\text{TF}528}$ (bearing a phenylalanine for tyrosine substitution at position 528, the regulatory phosphorylation site) resulted in improved antigen responsiveness, whereas the analogous *fyn*B product was much less effective (33). These results reinforce the idea that the p59^{fynT} isoform plays a fundamental role in TCR/CD3-mediated signaling.

Involvement of p59^{fyn} in B-cell signaling has also been suggested. Low levels of p59^{fyn} are detectable in normal B cells and in B cell lines, and the activity of p59^{fyn} is modestly increased following cross-linking of surface immunoglobulin (28, 37). Tables 1 and 2 list cell surface receptors that have been reported to associate with p59^{fyn}.

p56^{lck}

The product of the *lck* gene is expressed at high levels in T cells and at low levels in B cells, but in contrast to *fyn* appears exclusively in lymphoid cells, including thymocytes at all stages of maturation (21, 23, 38–40). Transcription of the *lck* gene is developmentally controlled through two distinct promoter elements separated in the human genome by 35 kb of germline DNA (39). The proximal promoter, positioned adjacent to the coding sequence, is active in thymocytes and is almost completely silent in peripheral T cells (39, 40). The 5′ or distal promoter, while active to some extent in thymocytes, exerts its principal effect in mature circulating T cells (39). Transcripts from both promoters encode identical proteins, hence the evolutionary conservation of these transcriptional regulatory elements

Table 1 Protein tyrosine kinase in lymphocytes

Kinase	Lineage	Physically associated primary receptor	Possible functions
fyn	T (21, 23)	TCR (31)	T cell activation
	B (21, 23)	Ig (28, 37)	B cell activation
	NK	CD23 (329)	NK cell activation
lck	T (21, 23, 38)	CD4, CD8 (48, 330, 331), IL2R (49)	T cell co-stimulation, IL-2R signaling, control of TCR gene rearrangement, early thymocyte proliferation
	B (21, 23, 38)	Ig (37)	B cell activation
lyn	B (21, 23)	Ig (28, 37, 83), IL-2R (85)	B cell activation, IL-2R signal
blk	B (23, 26)	Ig (28)	B cell activation
c-yes	T (23)	ND	?
CSK	T, B (90)	ND	Modulation of *src*-family kinase activity
ZAP-70*	T	TCR (5, 242)	T cell activation
syk	B (108)	Ig (110)	B cell activation
c-abl	T, B (94, 97, 99, 100)	ND	Transcriptional regulation (?)

* Catalytic function not yet established.

Table 2 *Lymphocyte receptors that stimulate protein tyrosine kinases*

	Receptors	Lineage	Kinase	Functions
Primary receptors	TCR	T	Zap70, *fyn*	T cell activation and proliferation
	Ig	B	*syk*, *blk*, *fyn*, *lck*, *lyn*	B cell activation and proliferation
	CD16	T/NK	ND	NK signal transduction
	CD23	B/NK	*fyn*	T/B accessory molecules
Accessory molecules	CD2	T	*fyn*, *lck*	Accessory molecules synergize with antigen receptor and stabilize intercellular interactions
	CD4	T	*lck*	
	CD5	T, B	*fyn*, *lck*	
	CD8	T	*lck*	
	CD19	B	ND	
	CD26	T	*lck*	
	CD28	T	ND	
	CD29	T	ND	
	CD40	B	ND	
Lymphokine receptors	IL-1	T, B	ND	T and B cell activation; synergizes with IL-2 and IL-4
	IL-2	T, B	*lck*	T and B cell activation
	IL-3	B	ND	Supports proliferation of hematopoietic cell precursors
	IL-4	T, B	ND	Proliferation and differentiation of T and B cells
	IL-5	B	ND	Induces murine B cell growth and differentiation
	IL-7	T, B	ND	Proliferation of pre-B cells and thymocytes; thymocyte differentiation

probably reflects the need to permit altered gene expression in response to differing external stimuli. For example, distal promoter-mediated transcription in human peripheral blood lymphocytes is downregulated in response to TCR/CD3 stimulation, following a temporal sequence that closely resembles, but is inversely correlated with, that which applies to accumulation of lymphokine transcript (41, 42). Mechanisms may also exist to specifically control $p56^{lck}$ translation (43, 44). Finally, lymphocyte activation provokes conversion of $p56^{lck}$ to forms with reduced electrophoretic mobility that are collectively designated $p60^{lck}$. This conversion is associated with, and may be determined by, increased serine phosphorylation at specific sites within the amino-terminal domain (45–47). Conversion of $p56^{lck}$ to $p60^{lck}$ follows stimulation of T cells with TCR ligands (45, 46, 48) or of T cells or NK cells with IL–2 (49, 50); the significance of this posttranslational modification remains obscure.

Four pieces of evidence suggest that $p56^{lck}$ participates directly in antigen-induced T cell activation. First, $p56^{lck}$ associates with the CD4 and CD8 co-receptors which contribute to antigen recognition. This association is mediated by a cysteine-containing motif, centered at residues 20–23 of the $p56^{lck}$ protein, which interacts with complementary cysteine-containing sequences in the cytoplasmic tails of the CD4 and CD8 co-receptor proteins (51, 52). Second, antibody-mediated cross-linking of CD4 or CD8 substantially increases the in vitro kinase activity of $p56^{lck}$ (53–55). This suggests that the CD4/CD8-$p56^{lck}$ complex may behave in a manner analogous to the EGF receptor, where ligand-induced dimerization stimulates increased protein tyrosine kinase activity (56–58). Third, infection of an insulin-specific mouse T hybridoma cell line with retroviruses encoding an activated form of $p56^{lck}$ markedly increased the sensitivity of cells to antigen as measured by IL–2 production (59) analogous to the experiments described with *fyn* retroviruses in the preceeding section. Thus $p56^{lck}$, like $p59^{fyn}$, can impinge upon a pathway that couples antigen recognition to T cell signaling. Mutagenesis experiments establish that all four domains of $p56^{lck}$ are required for satisfactory coupling of TCR-derived signals (60). Moreover, the $p60^{src}$ kinase proved incapable of mediating similar responses. Hence $p56^{lck}$ appears to couple to the TCR complex relatively specifically. Because the cell line used for these experiments lacked expression of both CD4 and CD8, the interaction with these co-receptor molecules must not be required for T cell signaling by $p56^{lck}$. Furthermore, because these experiments required constructs encoding the "activated" form (F505) of $p56^{lck}$, it is apparent that additional mechanisms exist to modulate the impact of $p56^{lck}$-mediated phosphorylation on T cell signaling.

A fourth set of experiments that argue for the importance of $p56^{lck}$ in

lymphocyte signal transduction employed a somewhat indirect approach. These studies made use of constructs that encode forms of CD4 with mutations in the p56^{lck} binding sequence. For example, Glaichenhaus and collaborators (61) demonstrated that in a T cell hybridoma which requires CD4 expression for antigen recognition, only those forms of CD4 that retained a p56^{lck} interaction motif proved capable of permitting satisfactory responses to antigen. Experiments performed in transgenic mice were even more revealing. In those studies, the *lck* proximal promoter directed 10-fold intrathymic overexpression of a CD4 minigene (62). Remarkably, the presence of excess CD4 protein depleted p56^{lck} from CD8 binding in CD4$^+$8$^+$ thymocytes (63). This substantially compromised intrathymic selection of cells bearing a class I–restricted TCR (34, 62). Overexpression of a mutant form of CD4 lacking the cytoplasmic tail had no such effect (63). Thus, in contrast to p59^{fyn}, p56^{lck} is expressed in thymocytes undergoing selection and appears to participate in repertoire selection. However, these studies are by no means definitive, and indeed a recent report indicates that protein tyrosine kinase inhibitors do not block negative selection of thymocytes (modeled in vitro) even though p56^{lck} activity is rendered undetectable (64).

More direct evidence for the involvement of p56^{lck} in thymocyte selection might in principle derive from manipulation of *lck* expression in transgenic mice. However, thymocytes have proven to be exquisitely sensitive to alterations in p56^{lck} activity, which may in part explain the existence of the many regulatory mechanisms, outlined above, that modulate p56^{lck} expression. For example, mice bearing a targeted disruption of the *lck* gene manifest severe defects in T cell maturation, with a 100-fold reduction in the number of thymocytes, and an overall failure in T cell development (65). Similarly, mice expressing a catalytically inactive form of p56^{lck} at high levels fail to generate mature T lymphocytes (S. D. Levin, S. J. Anderson, K. A. Forbush, R. M. Perlmutter, *EMBO J.*, manuscript in press). In the latter case, a discrete block in thymocyte development exists at the point where CD3$^-$4$^-$8$^-$ blast cells replicate and undergo a chain gene rearrangement to emerge as CD4$^+$8$^+$ cells (66, 67). Thus, p56^{lck} activity is absolutely required for normal thymocyte development. Intriguingly, even modest expression of the "dead" p56^{lck} produced significant thymic abnormalities, and mice heterozygous for an *lck*$^-$ allele also exhibited modest T cell abnormalities (65). Hence normal levels of p56^{lck} must be rigorously maintained to permit T cell maturation.

Overexpression of p56^{lck} produces even more deleterious effects, ranging from arrested development of thymocytes to frank thymic malignancy (68, 69). High levels of p56^{lck} block T cell development by specifically interdicting Vβ to Dβ joining and hence preventing expression of T cell

receptors (S. J. Anderson, K. M. Abraham, T. Nakayama, A. Singer, R. M. Perlmutter, *EMBO J.* 11: 4877). The mechanism by which $p56^{lck}$ exerts this effect is under investigation; however, experiments using mutant forms of an *lck* transgene make plain that interaction with CD4 and CD8 is not required for $p56^{lck}$ to act on the fidelity of TCR gene rearrangement (S. D. Levin, K. M. Abraham, K. A. Forbush, R. M. Perlmutter, manuscript in preparation).

Unfortunately, these studies provide no information on the role of $p56^{lck}$ in transmitting signals from the T cell antigen recognition complex, or on the importance of $p56^{lck}$ in mediating T cell repertoire selection. It appears possible that $p56^{lck}$ may perform different functions in T lineage cells at different stages of differentiation. Further progress in this field awaits the development of selective means to manipulate expression of $p56^{lck}$ in both developing and mature T cells.

Several reports have demonstrated that association of $p56^{lck}$ with co-receptors facilitates the responsiveness of hybridomas when the appropriate co-receptor is also included in the activation complex either by antigen-induced activation or by co-cross-linking of TCR and CD4 or CD8 (61, 70–72). This enhancement is dependent on the ability of the co-receptor to associate with $p56^{lck}$ (61, 71, 72). Because CD4 and CD8 are not always required for T cell activation, it is possible that $p56^{lck}$ serves as a signal amplifier rather than as an obligatory activator, or that other ways of including it in the activation complex exist.

One indication that $p56^{lck}$ may play a fundamental role in T cell activation is the discovery of an lck^- mutant of the Jurkat cell line (73). This mutant fails to produce functional $p56^{lck}$ and does not mobilize intracellular calcium in response to stimulation by anti-CD3 antibody despite expression of a normal TCR/CD3 complex and of $p59^{fyn}$. The calcium response was reconstituted by transfecting the cells with a functional *lck* gene. A mouse CTLL T cell line lacking $p56^{lck}$ expression has also been described and exhibits defects in TCR-mediated cytotoxic activity (74).

These experiments demonstrate that $p56^{lck}$ can influence thymocyte development and TCR signaling. In addition, $p56^{lck}$ specifically associates with the β chain of the IL–2 receptor and becomes activated following treatment of human peripheral blood T cells or NK cells with IL–2 (49, 50). Association between $p56^{lck}$ and the IL–2Rβ chain depends on the acidic region of the β chain cytoplasmic domain, and on sequences within the NH_2-terminal half of the kinase domain of $p56^{lck}$ (49). Recent studies demonstrate that association of the kinase with the receptor is, by itself, insufficient to permit IL–2–mediated kinase activation. Sequences within the membrane-proximal serine-rich region of the IL–2Rβ chain are also

required (75–77). Moreover, this same serine-rich region defines the regulatory element that controls the transmission of IL–2–mediated proliferative signals in cell lines made IL–2–responsive through transfection of IL–2Rβ chain expression constructs (76). These data suggest that p56^{lck} contributes to, but is not required for, IL–2R signal transduction. More recent studies indicate that activation of p56^{lck} by IL–2 helps to control expression of the transcriptional regulatory complex encoded by the c-*fos* and c-*jun* genes (77).

In summary, it is apparent that p56^{lck} subserves numerous functions at various stages of lymphocyte development. It interacts with at least three extracellular receptors (CD4, CD8, and the IL–2Rβ chain) and probably with others as well (Table 1). At least some p56^{lck} functions appear to be unique, so that animals lacking p56^{lck} fail to elaborate normal T cells. Other p56^{lck} functions may overlap those of related kinases. Despite substantial information on the importance of p56^{lck} in normal T cell differentiation, substrates for p56^{lck} activity have not been unambiguously identified. In LSTRA cells, a mouse lymphoblastic cell line which contains high levels of activated p56^{lck}, constitutive phosphorylation of *ras*-GAP is observed (78), and other studies suggest that phospholipase Cγ1 (60, 79) and the phosphatidylinositol–3′ (PI–3) kinase (80) may be targets of p56^{lck} activity (Figure 2). However, a definitive link between p56^{lck} function and the activity of any of these putative substrates has yet to be established. The possible involvement of p56^{lck} in regulating MAP kinases is described in more detail below.

p53/56^{lyn}

The product of the *lyn* gene is found in B cells and B cell lines, in a number of nonhematopoietic tissues, and in macrophages, monocytes, and platelets, but not at appreciable levels in T cells (21, 23). Two isoforms of the *lyn*-encoded protein have been identified (p53^{lyn} and p56^{lyn}), the products of distinct transcripts derived through variable utilization of an intra-exonic splice donor sequence (81, 82).

Cross-linking of surface immunoglobulin (Ig) in B cells induces accumulation of phosphotyrosine (28, 37), so it is notable that both forms of *lyn*-encoded protein have been found associated with surface Ig in immunoprecipitates from B cell lines and from splenic B cells (28, 37, 83). However, similar results can be obtained for other *src*-family kinases (see Table 1). Cross-linking of surface Ig, which normally activates B cells, increases the in vitro kinase activity of p53/56^{lyn} (28, 37, 84), but again similar results were obtained when other *src*-family members were analyzed. Interestingly, stimulation of B cells with activating doses of LPS has little effect on *src*-family kinases, including p53/56^{lyn} (28), suggesting the existence of

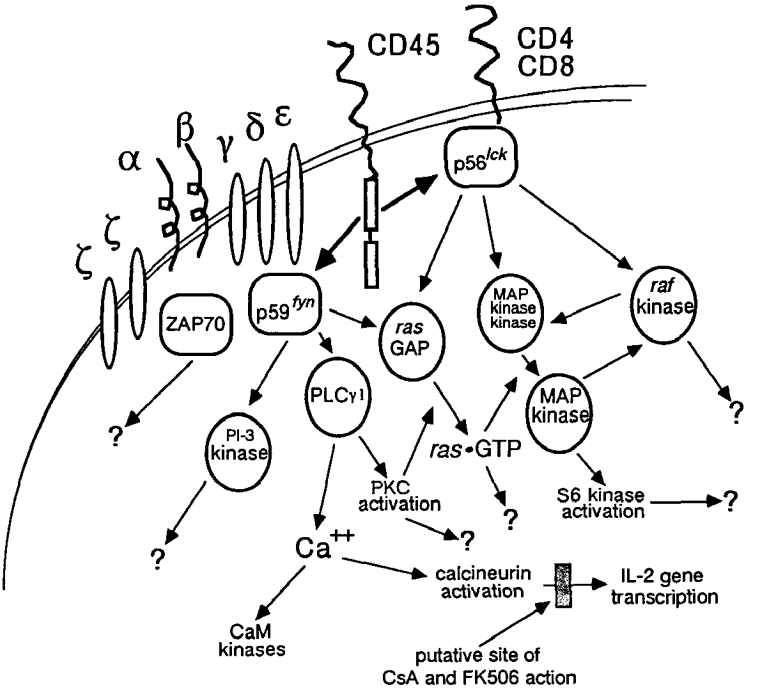

Figure 2 A schematic diagram linking T cell antigen recognition to biochemical signaling pathways. The CD45 PTPase, along with the CD4/CD8-p56^{lck} coreceptor, assists the CD3-p59^{fyn} signaling complex in regulating a second, and as yet incompletely defined, set of command and control molecules. Those listed here have been noted to be either phosphorylated or activated following TCR/CD3 stimulation. The arrows in each case define potential regulatory influences. Definitive evidence supporting the pathways outlined here remains to be adduced. Instead, the diagram serves to illustrate the complex nature of the regulatory circuits that must be elucidated in order to make sense of lymphocyte signal transduction. See text for discussion.

alternative activation pathways. Further evidence that p53/56^{lyn} participates in the B cell antigen receptor signaling complex was recently provided by the demonstration that the p85 regulatory subunit of the PI–3 kinase associates with p53/56^{lyn} after cross-linking of Ig, and that PI–3 kinase activity was also present in anti-Ig immunoprecipitates (84).

As discussed previously, signalling by the IL–2 receptor may involve p56^{lck} in T cells, but B cells and some B cell lines respond to IL–2 despite the presence of little or no p56^{lck}. Torigoe and co-workers (85) recently described the activation of p53/56^{lyn} in response to IL–2 in a pro-B cell line. IL–2 failed to activate p59^{fyn} and p62$^{c\text{-}yes}$, the other *src*-family kinases

present. In addition, p53/56^{lyn} was shown to be associated with the IL–2Rβ chain suggesting that this kinase may subserve functions in B cells that are attributable to p56^{lck} in T cells. Transfection studies in COS cells with constructs encoding chimeric kinases with the NH_2-terminal, SH3 and SH2 domains of p56^{lck} coupled to various kinase domains indicate that *src*-family kinases in general can probably associate satisfactorily with the IL–2Rβ chain (49, 86). The importance of these associations remains unassessed.

p62$^{c\text{-yes}}$

Like p59^{fyn}, p62$^{c\text{-}yes}$ is found in a variety of tissues including peripheral T cells and thymocytes (23). However, the levels of p62^{yes} in lymphoid cells are quite low, and there exist no data to support its role in lymphocyte signaling. The *yes*-encoded protein may participate in platelet signal transduction pathways (87, 88).

p50^{csk}

The product of the *csk* gene is related to *src*-family kinases in both form and function. Originally identified through purification of an activity in rat brain that specifically phosphorylated the carboxy-terminus of p60^{src} (89), subsequent cDNA cloning permitted characterization of a molecule with *src*-like primary structure but lacking a myristylation signal, autophosphorylation sites, or a C-terminal regulatory tyrosine phosphorylation site (90). The enzyme is found in both the cytosolic and membrane fractions in rat brain (90). Purified p50^{csk} from neonatal rat brain phosphorylates the COOH-terminal tyrosines of *src*-family kinases generally (91). This observation, coupled with the high-level expression of *csk* transcripts in lymphoid tissues (90), suggests that p50^{csk} may be a fundamental regulator of the activity of *src*-family kinases in lymphocytes. Experiments evaluating this hypothesis have not yet appeared.

c-abl

The product of the c-*abl* gene has been intensely studied for nearly a decade (92–96), but little is known of its normal function. The protein includes an NH_2-terminal PTK domain, an SH2 and an SH3 domain, and a long COOH-terminal tail of undefined function (97). Transforming versions of the *abl*-encoded kinase virtually always elicit hematopoietic tumors, suggesting that this kinase can impinge upon proliferation control mechanisms that predominate in these cells (98). Although it is expressed in most tissues and cell types, highest levels of p150$^{c\text{-}abl}$ are found in spleen and thymus (94, 97, 99, 100). Several forms of the c-*abl*-encoded protein exist, the products of differentially spliced mRNAs (101). One form (Type

IV) is myristylated and localizes within the nucleus (102, 103). At least two studies indicate that $p150^{c\text{-}abl}$ has DNA binding activity (104, 105). One of these concludes that the c-*abl* gene product binds to a specific viral enhancer DNA sequence (104), suggesting a role for this kinase in transcriptional control.

Targeted disruption of the c-*abl* gene yields small, sickly mice which exhibit impaired T and B cell development, perhaps reflecting the normally high level of expression of c-*abl* in those compartments (106, 107). However, the severity of this recessive genetic defect renders interpretation difficult, since stress and nutritional deficits can profoundly affect lymphocyte development.

p72^{syk}

A PTK of 72 kDa has been purified from the cytosolic fraction of porcine spleen, and its corresponding cDNA was subsequently cloned and sequenced (108). This molecule (designated $p72^{syk}$) also has a COOH-terminal kinase domain like members of the *src*-family, but it lacks an SH3 domain and has two SH2 domains in the amino-terminal half. It also lacks a myristylation signal and boasts no membrane spanning domain (108). Wheat germ agglutinin, which exerts predominantly stimulatory effects on B lymphocytes, provokes increased phosphotyrosine accumulation in splenocytes and also increases the kinase activity of $p72^{syk}$ about 2.5-fold (109). More recently a 72-kDa PTK expressed predominantly in B cells has been found associated with the B cell antigen receptor. This kinase becomes activated and tyrosine-phosphorylated following cross-linking of Ig (110). The susceptibility of this protein to proteolysis suggests that it represents $p72^{syk}$ (108–110). Interactions among $p72^{syk}$ and the *src*-family kinases, which in B cells also associate with the surface immunoglobulin complex, remain unexamined.

p72^{emt} *and p77*^{emb}

Two novel receptor PTKs expressed primarily in B-lineage ($p77^{emb}$) or T lineage ($p72^{emt}$) cells have recently been identified (111). Structurally similar to the drosophila Dsrc28 kinase, these kinases contain SH2 and SH3 domains, and they share a unique amino-terminal motif containing basic amino acids followed by a short proline-rich segment. The profound cell-type specificity of these PTKs suggests that they contribute to regulatory pathways that exist only in hematopoietic cells.

PROTEIN SERINE/THREONINE KINASES

Although receptor-type protein serine kinases have been described (112), the bulk of these molecules, which carry out >95% of all phosphorylation

events in all cells, are intracellular proteins that integrate signals from many sources. Over the years, researchers studying protein phosphorylation have come to accept the serine/threonine kinases as molecules engaged in cell regulatory circuits common to all cell types. In contrast to the protein tyrosine kinases, many serine/threonine kinases act on very well-defined substrates, and the regulation of some of these molecules has been extensively examined. Space does not permit an exhaustive catalogue of protein serine/threonine kinases in lymphocytes. However, we have endeavored to include those for which some evidence linking their activity to lymphocyte functions has been adduced.

Protein Kinase C

Protein kinase C (PKC) was originally described as a serine/threonine protein kinase activated by limited proteolysis with calpain, although it was later shown that it was dependent on calcium and phosphatidylserine, and that diacylglycerol greatly increased its affinity for these co-factors, thereby activating PKC at basal calcium concentrations (113, 114). The role of PKC in lymphocyte activation is suggested by the ability of phorbol esters (which bind and activate PKC—115) plus calcium ionophore (providing a surrogate TCR signal) to activate lymphocytes, whereas ionophore by itself is ineffective (116). Because stimulation of TCR/CD3 augments phosphatidylinositol-specific phospholipase C (PLC) γ activity leading to the generation of diacylglycerol and phosphoinositides, transient stimulation of PKC, which can be demonstrated experimentally, has been proposed to assist in propagation of the T cell activation sequence (117–120).

Eight genes encoding nine different forms of PKC denoted α, βI, βII, γ, δ, ε, ζ, η, and θ (119, 121, 122; A. Altman, personal communication) can be divided into two structurally related groups. All share a kinase domain formed by two conserved regions C3 and C4, and a region in the regulatory domain, C1. However, the δ, ε, ζ, η, and θ isoforms lack a second conserved region (C2), common to the other four PKC isotypes. This domain seems to be involved in calcium binding (123), and the δ, ε, ζ, and η isoforms are not dependent on calcium for activation (122, 124, 125).

Since the different isoforms have different requirements for activation and may have different substrate specificities, activation of discrete isozymes could variably influence lymphocyte signaling. Although many studies address the pattern of expression of the PKC isoforms in lymphocytes, the results to date are somewhat contradictory. Most agree that the α, β, δ, ζ and η isoforms are generally present in lymphocytes (122, 126–132). However, controversy exists regarding the ε form (124, 125, 130, 132, 133).

Although stimulation of lymphocyte antigen receptors activates PKC (119, 134), lymphocyte proliferation ordinarily does not result, probably

because the activation of PKC in such cases is transient when compared with that induced by phorbol esters (134, 135; reviewed in 120). The generation of diacylglycerol following antigen receptor stimulation may result from stimulation of PLCγ (itself perhaps controlled by tyrosine phosphorylation—136), via activation of phospholipase D (137), or through other mechanisms (138).

Several other accessory molecules may also direct activation of PKC, as in the case of CD2 (139, 140). CD2 stimulation may also augment intracellular arachidonate concentrations (141) which can synergize with diacylglycerol to activate PKC α and β (142). Stimulation with CD5-specific mAbs may increase CD3-induced phospholipid turnover (143), and in some cases activates PKC independently of phosphatidylinositol or phosphatidylcholine hydrolysis (144). It has also been proposed that IL–1 stimulates production of diacylglycerol by hydrolysis of phosphatidylcholine (145). These mechanisms could act together to permit the sustained activation of PKC required for lymphocyte proliferation.

Although the importance of PKC in lymphocyte activation seems clear, its substrates remain largely undefined. Research addressing this question depends on the use of PKC activators (e.g. phorbol esters), PKC inhibitors (e.g. H–7 or staurosporine), or the generation of PKC-deficient cells through prolonged exposure to phorbol esters. Such experiments inevitably suffer from the incomplete specificity of the pharmacological agents employed. The characterization of an endogenous pseudosubstrate, which is a highly specific PKC inhibitor (146), has proved helpful in examining the role of PKC in the phosphorylation of the CD3γ chain (147) and in the activation of p21^{ras} after stimulation of T cells through CD3 (148). Downregulation of PKC by prolonged treatment with high doses of phorbol esters cannot be achieved in all cells (134, 147), and some PKC isoforms [e.g. ζ (149) and ε (133)] are refractory to this treatment. Nevertheless, when treated in this way, some T cell lines no longer couple the TCR/CD3 complex to the IL–2 gene, perhaps because they fail to activate AP–1 expression (150).

Table 3 lists some aspects of cell physiology believed to be regulated by PKC; however, the importance of PKC-mediated phosphorylation is in no case securely established. For example, although there is a correlation between phosphorylation of CD3 and CD4 and their downregulation of these receptors (151, 152), published data support contrary positions regarding the impact of of phosphorylation itself (153, 154). Similarly, although PKC phosphorylates the β chain of LFA–1, the serine phosphorylation events, per se, may not actually affect LFA–1/ICAM–1 binding, and stimulation through CD3 increases LFA–1 affinity by both PKC-dependent and PKC-independent mechanisms (155).

Table 3 Possible functions of serine/threonine kinases in lymphocytes

Kinase	Expression T cells	Expression B cells	Possible function
PKC α	+	+	Regulation of adhesion (155)
PKC β	+	+	Cytoskeletal reorganization (332–334)
PKC γ	−	−	Induction of gene expression (114, 121)
PKC δ	+	+	Down-regulation of surface receptors (151, 152, 335, 336)
PKC ε	+/−	+/−	Regulation of activation
PKC ζ	+	+	Regulation of *raf* (157)
PKC η	+	+/−	Regulation of p21^{ras} (156) Regulation of PLC-γ1 (163)
Erk-1	+	+	Cell cycle control (184–188)
Erk-2	+	+	
RAF-1	+	+/−	
RAF-A	+/−	+/−	
RAF-B	−	−	
PKA	+	+	Inhibition of T cell activation at different levels: CD3-induced PI metabolism (165–167, 337) CD3-ζ tyrosine phosphorylation (169) IL2R expression (176, 177) IL2 activation pathway (178) IL2 gene expression (173–175, 338) Inhibition of cell-cell adhesion (155, 179)
p70S6K	+	?	Cell cycle control (230, 339, 340)
PKG	+	+	
Ca^{+2}-CaM-dep K	+	+	

PKC may regulate several TCR-induced secondary responses. Among them, the activation of p21^{ras} (156), phosphorylation and regulation of the activity of *raf* kinases (157), and the activation of the MAP kinases (158, 159), perhaps by regulating the MAP kinase activator or MAP kinase kinase (160). Although PKC phosphorylates p56^{lck} (45, 47), no effect on its catalytic activity has been shown.

Activation of PKC may also exert a negative effect on T cell activation, in that pretreatment with phorbol esters can inhibit the phospholipid hydrolysis induced by stimulation through TCR/CD3 (161, 162). This effect may reflect PKC-mediated phosphorylation of PLCγ1 (163).

cAMP-Dependent Kinase

Although there exists a huge literature on the effects of cAMP on lymphocyte activation (119, 164), effects which almost certainly reflect changes

in cAMP-dependent protein kinase (PKA) activity (see 164 for additional discussion), the physiological substrates of PKA in lymphocytes remain mostly unknown. In general, treatment of lymphocytes with agents that increase intracellular cAMP levels inhibits their activation. This blockade occurs early in the antigen-receptor stimulation pathway, because membrane phospholipid turnover, ordinarily an early event, is blocked (165–167). This could result from direct phosphorylation and inhibition of PLCγ1 activity (163, 168), or through inhibition of a protein tyrosine kinase, perhaps the one that phosphorylates CD3ζ (169). In contrast, activation of PKA had no effect on PI metabolism induced in B cells by anti-Lyb–2 (165). Some authors find that augmented concentrations of intracellular cAMP block proliferation induced by ionophores and phorbol esters (170–172), agents which together should promote activation in the absence of alterations in membrane phospholipid turnover. Hence PKA may act at several different points during lymphocyte stimulation. For example, cAMP may block the IL–2/IL–2R system by suppressing transcription of the IL–2 gene (173–175), by modifying expression of IL–2R (176, 177), and by interfering with IL–2–stimulated phosphorylation (178). Agents that increase cAMP concentrations are also able to inhibit CD3-, CD7-, and CD28-induced lymphocyte adhesion (155, 179).

Under what circumstances might augmented cAMP concentrations accumulate? Both T and B cells express receptors for prostaglandin E (180), and increases in cAMP induced by this inflammatory mediator could play a role in the regulation of lymphocyte responsiveness. Activation through CD3 (181), CD2 (182), and, in some cases, CD29 (183) (VLA–4) may also increase intracellular cAMP concentrations, although the physiological significance of these increases is unknown.

MAP Kinases

Mitogen-activated protein kinases (MAP kinases), also known as *erks* (extracellular regulated kinases), are a group of protein serine/threonine kinases that respond to serum growth factors, phorbol esters, nicotinic acid, and many other stimuli (184–188). These proteins were first identified as kinase activities in extracts of insulin-stimulated 3T3 fibroblast that phosphorylated microtubule-associated protein 2 (189). In mammals, three different MAP kinase cDNAs have been cloned: *erk*–1, which encodes $p44^{mapk}$, *erk*–2 ($p42^{mapk}$), and *erk*–3, the product of which, predicted to migrate as a 63-kDa protein, remains uncharacterized (190–192). Some antisera raised against the COOH-terminal residues of $p44^{mapk}$ also recognize a 45-kDa protein (*erk*–4 kinase) for which cDNA clones do not yet exist (192). Transcripts corresponding to all known *erk* mRNAs can be

detected in thymocytes, and the products of *erk*1 and *erk*2 have also been detected in human peripheral blood T cells by immunoblotting (193). Stimulation of peripheral blood lymphocytes or Jurkat cells with anti-CD3 mAbs, anti-CD2 mAbs, or phorbol esters induces activation of MAP kinases (158, 193–195). Phorbol esters also induce MAP kinase activity in EL–4 cells, a murine thymoma cell line (196).

Activation of MAP kinases requires phosphorylation at both tyrosine and threonine residues (197, 198) that are localized within a single short peptide sequence (199). PKC can regulate MAP kinase activity, as illustrated by experiments employing phorbol ester agonists, however PKC-independent mechanisms of MAP kinase activation also exist (200–202). Relevant to lymphocyte signaling, $p56^{lck}$ was found to phosphorylate and activate $p44^{mapk}$ purified from sea star oocytes in vitro (203). Futhermore, coaggregation of CD3 and CD4 enhances CD3-mediated activation of MAP kinase (159), while in $CD4^-$ T cell hybridoma variants, stimulation with CD3 does not stimulate MAP kinase activity (203). These results suggest that $p56^{lck}$ could participate in the control of MAP kinase function in T cells. However, it is important to note that the sea star enzyme differs from mammalian MAP kinases in its regulatory sequences. $p21^{ras}$ may also regulate MAP kinases in lymphocytes, as has been recently shown in other cell systems (204–206).

Following in vitro dephosphorylation, MAP kinases undergo autophosphorylation on both tyrosine and threonine residues (207), events that correlate with the development of increased kinase activity toward exogenous substrates. The functional significance of this mode of regulation remains speculative. Potential MAP kinase regulators have been identified in lysates of stimulated 3T3 fibroblasts (208) or PC–12 cells (209). These factors reactivate dephosphorylated MAP kinases in the presence of ATP, and recent experiments make plain that the kinase activator is itself a kinase (MAP kinase kinase), which is also regulated by serine/threonine phosphorylation (160, 210). Whether this MAP kinase kinase provides the only mechanism of regulation of MAP kinase activity remains untested.

Recent hypotheses focus on the role of MAP kinases in promoting the G0 to G1 transition. Several potential MAP kinase substrates have been identified in vitro, and a consensus sequence for the phosphorylation target has been proposed. Among the putative substrates are the *raf* and 90-kDa *rsk* serine/threonine kinases (211–213), the AP–1 nuclear factors encoded by the c-*jun* (214) and c-*fos* (215) genes, and the c-*myc* gene product (216). Interestingly, recent data also suggest that the *raf*-encoded kinase may directly activate the map kinase kinase (217), making $p72^{raf}$ an activator of a cascade which ultimately acts reflexively.

*p72*raf

The *raf*–1 protooncogene encodes a cytoplasmic serine/threonine specific protein kinase of 72 kDa involved in signal transduction from the cell surface to the nucleus in different cell systems (see 218–220 for recent reviews). Three mammalian *raf* genes have been identified, *raf*–1, A-*raf*, and B-*raf*. Low levels of *raf*–1 and A-*raf* mRNA appear in murine lymphoid tissues (221), but B-*raf* is not expressed in lymphoid cells. Transcripts encoded by *raf*–1 also accumulate in resting human T cells (222), and some experiments suggest that *raf*–1 expression in lymphocytes increases during activation (222, 223).

The activity of *raf*-encoded proteins is itself regulated by phosphorylation (218, 219)). In the 2B4 mouse T cell hybridoma, stimulation with anti-CD3 or anti-Thy–1 mAbs increases both the activity and the extent of phosphorylation of *raf*–1 kinase through a PKC-dependent mechanism (157). Normal peripheral T cells, though responsive to phorbol esters, apparently do not support *raf*–1 kinase activation following stimulation with anti-CD3 antibodies (222). In B cells, cross-linking of surface immunoglobulins increases $p72^{raf}$ kinase activity and promotes phosphorylation of $p72^{raf}$ kinase on serine residues (224). A recent report indicates that activation of $p56^{lck}$ induces transient serine and tyrosine phosphorylation of $p72^{raf}$ to a very small extent, as well as association of a tiny fraction of $p72^{raf}$ kinase (1% of the total) with the CD4-$p56^{lck}$ complex (225). Activation of *raf* kinases following IL–2 treatment has been reported in mouse CTLL–2 cells (226) and in human lymphoblasts (222). This effect may also reflect $p56^{lck}$ activation, since IL–2 treatment dramatically augments phosphate transfer by this protein tyrosine kinase. The MAP kinases may serve as intermediaries in this signaling process (212; see Figure 2).

S6 Kinases

Two families of serine/threonine protein kinases capable of phosphorylating the S6 ribosomal protein have been described (227). Whether this phosphorylation event actually influences translation remains controversial. The 90-kDa *rsk*-encoded S6 kinases are activated in response to T cell receptor stimulation (228), perhaps via a pathway involving the MAP kinases (213). Whereas both the p70 S6 kinase and the $p90^{rsk}$ S6 kinases are activated by polypeptide growth factors in mouse fibroblast lines (229), IL–2 induces p70 S6 kinase activity selectively. This response can be blocked by rapamycin (230). Hence it is possible that the S6 kinases may prove to be important selective regulators of mitogenesis mediated by different T and B cell surface receptors.

LYMPHOCYTE RECEPTORS THAT INTERACT WITH PROTEIN KINASES

As previously discussed, B cell surface immunoglobulin, the TCR/CD3 complex, CD4 and CD8 co-receptor molecules, and the IL–2 receptor all interact productively with intracellular protein tyrosine kinases and may in many cases interact with the same kinase molecules. Hence one challenge for those attempting to dissect the linkage of lymphocyte cell surface receptors with the cell interior is to ascertain how degenerate signaling pathways can elicit quite specific effects. The problem becomes more challenging when the full range of receptor structures that stimulate heightened accumulation of phosphoproteins is tabulated.

T Cell Receptor Complex

The α and β chains of the TCR each contain a single membrane-spanning domain and a very short cytoplasmic region. In contrast, the associated CD3 subunits contain large intracytoplasmic domains and act to permit receptor assembly and signal transduction (231). A short sequence motif positioned within the cytoplasmic domains of the three closely related members of the complex, the γ, δ, and ε subunits, and both the CD3ζ and η polypeptides (products of alternative splicing of the ζ gene) serve to couple these molecules to signal transducing structures (232, 233). The ζ chain boasts a six-residue extracellular domain, is homologous to the γ chain of the basophil and mast cell high affinity IgE receptor (FcεRI), and is associated as well with CD16 in NK cells (234 and references therein). In T cells, the ζ chain becomes rapidly phosphorylated after receptor engagement, and its intracellular domain contains multiple sites for tyrosine phosphorylation. However, recent studies make plain that mutations in these phosphorylation sites do not compromise signaling appreciably (235). While initial reports proposed that signaling via $\zeta\zeta$ and $\zeta\eta$ complexes (the commonly observed disulfide-linked forms) might differ (236, 237), subsequent studies failed to support this view (238). Reconstitution of signaling in ζ chain-deficient cells has been achieved using either ζ chain expression constructs or chimeric proteins in which the cytoplasmic domain of ζ was fused to the extracellular and transmembrane domains of CD4, CD8, or the IL–2Rα chain. In the latter cases, antibody-mediated cross-linking of the chimeric receptors provoked a typical T cell activation sequence (233, 239–241). Two groups (242, 243) have recently described a 70-kDa protein (Zap 70) that becomes phosphorylated on tyrosine and associates with the ζ chain upon TCR stimulation. Zap70 immunoprecipitates contain a kinase activity, and it is plausible that Zap70 is itself a protein tyrosine kinase.

The γ, δ, and ε subunits of the CD3 complex are phosphorylated on serine after receptor engagement, but the significance of these phosphorylation events is obscure. Tyrosine phosphorylation events mediated by chimeric receptors bearing ζ- or ε-derived coupling motifs reportedly differ (241), suggesting that different signaling elements may couple to the different CD3 subunits. The importance of *src*-family kinases in these signaling pathways has already been discussed.

Accessory Receptor Structures

A number of cell surface molecules that are incapable of providing a primary stimulus for T cell activation nevertheless transduce signals that synergise with those derived from the TCR/CD3 complex. Under certain conditions, these accessory molecules may also be capable of directing the mobilization of intracellular calcium stores or stimulating the entire activation response (see 119 for a review). Not surprisingly, these accessory molecules also provoke changes in protein phosphorylation.

CD2

The CD2 co-receptor molecule, a 50-kDa lymphocyte surface protein, promotes cellular adhesion by binding LFA–3, a glycoprotein present on epithelial and endothelial cells and on connective tissue (244). CD2 also provides costimulatory signals in CD3-induced T cell proliferation. That this synergy involves more than the stabilization of intercellular interactions is suggested by the observation that certain combinations of anti-CD2 reagents can independently induce T cell proliferation, a response potentiated by simultaneous provision of anti-CD5 (see 245 for review). The stimulation of thymocytes through CD2 recapitulates those events that occur after engagement of the TCR/CD3 complex, promoting the mobilization of intracellular calcium stores and the phosphorylation of a number of proteins, including PLCγ1 (246, 247). Stimulation with anti-CD2 mAbs requires a functional T cell receptor: CD2 is incapable of signaling in CD3-TCR-deficient mutants of the Jurkat cell line, but becomes competent after TCR/CD3 reconstitution by transfection of deficient TCR subunits. In particular, this reconstitution requires the presence of a functional ζ chain with an intact cytoplasmic domain (248). In this instance there is no evidence that clones expressing a ζ chain truncated in its cytoplasmic domain retain the potential to signal through ε after CD2 stimulation. This may indicate a specific association between the cytoplasmic tails of CD2 and CD3ζ. CD2-immune complexes prepared from BRIJ–96 lysates of rat T cells contain p56^{lck} and p59^{fyn} (249), and anti-CD2 reportedly activates p56^{lck} in mouse T cells (250).

CD28

CD28 is a 44-kDa glycoprotein expressed as a homodimer on the surface of all $CD4^+$ and about half of $CD8^+$ human T cells, and on most mouse T cells (251, and references therein). Stimulation of T cells with anti-CD28 antibody provides an accessory activation signal, enhancing the production of lymphokines and increasing T cell proliferation as a consequence of stimulation by other ligands. In addition, the cross-linking of CD7 and CD28 in human T cells induces integrin-mediated adhesion to fibronectin, ICAM–1 and VCAM–1 via a kinase-dependent mechanism (179). One ligand for CD28 has been identified as the cell surface glycoprotein B7/BB1 which is present on activated B cells and IFN-γ-stimulated monocytes (252). Ligand occupancy of CD28 receptors recapitulates those events observed after monoclonal antibody activation (253–256).

Vanderberghe and colleagues (257) recently observed that responses to CD28-B7/BB1 ligation proceed in cells lacking CD3 expression. Biochemical evidence also suggests that the pathways emanating from the CD28 receptor differ from those invoked by the TCR because TCR-mediated signal transduction events are sensitive to cyclosporine and to inhibitors of protein kinase C, while T cell stimulation through CD28 remains unaffected by these agents (258–260). However, both signals are sensitive to tyrosine kinase inhibitors, and patterns of phosphotyrosine accumulation observed following stimulation through CD28 and the TCR contain similar elements (257, 260).

Glycosylphosphatidylinositol-Linked Membrane Proteins

A number of lymphocyte surface antigens which are anchored in the membrane by glycosylphosphatidylinositol (261) may also serve as accessory molecules. These include the CD59 (T cell activating protein, TAP), CD55 (decay accelerating factor), CD48, CD24, CD14, and Thy1 proteins. Binding of ligand to these molecules can induce lymphocyte activation and the phosphorylation of proteins on tyrosine (262, 263). Moreover, $p56^{lck}$ activity can be detected in immune complexes formed with CD59, CD55, and CD48 in human T cells, and with Thy1 in mouse T cells (262, 263). However, enthusiasm for the significance of these associations is undermined by the extraordinary abundance of molecules such as Thy1 and $p56^{lck}$ in T cells, and the mechanism whereby these GPI-anchored, external molecules might be coupled to intracellular kinases is unclear.

B Cell Antigen Receptor

The Ig present on the surface of B lymphocytes functions analogously to the T cell receptor: engagement of surface Ig by antigen (or by anti-Ig

antibodies) induces a series of metabolic events, culminating in the activation of resting B cells, their entry into the cell cycle, and the internalization of antigen which is subsequently processed for presentation to T lymphocytes. Receptor engagement induces the phosphorylation of a number of proteins on tyrosine, including PLCγ2 (PLCγ2 is the most abundant form of PLCγ in B cells, whereas T cells express PLCγ1), implicating one or more protein tyrosine kinases in these processes (264–269). Tyrosine kinase inhibitors block the process of Ig receptor internalization (270) and disrupt those phosphorylation events that normally occur as a consequence of cross-linking of other B cell surface signaling molecules, including CD19/CR2, CD20, CD39 and CD40 (267, 271). Candidates for this kinase activity include the *src* family tyrosine kinases $p55^{blk}$, $p53/56^{lyn}$, and $p59^{fyn}$, all of which are expressed in B cells (21, 26, 27). These kinases appear in immunoprecipitates formed using anti-IgM or anti-IgD (28, 84), and their activities increase following anti-Ig stimulation (28, 84). The availability of animal models in which expression of these kinases has been manipulated should permit the dissection of these signaling pathways.

Interleukin Receptors

The members of a novel superfamily of interleukin receptors which includes the IL–2Rβ chain, the IL–3, IL–4, IL–5, IL–6, and IL–7 receptors, the gp130 component of the IL–6, LIF and oncostatin M receptors, the GM-CSF, G-CSF, and erythropoietin receptors, and the growth hormone receptor, all of which share common structural features in their extracellular domains, have recently been described (272). Considerable variation exists in the size and nature of the cytoplasmic chains of these receptors, suggesting that disparate signaling pathways emanate from the individual members of the family, and the nature of these pathways remains obscure. Detailed reviews of the cytokine receptor superfamily have recently appeared (273); we confine our discussion to consideration of the role of phosphorylation events in signaling by members of this group. The best analyzed case, that of the IL–2Rβ chain, was discussed.

IL–4 Receptors

IL–4 is active in both T lymphocytes and B cells, where it synergizes with nonmitogenic concentrations of anti-Ig antibodies or phorbol esters to stimulate DNA synthesis and entry into the cell cycle (274). The molecular events occurring after stimulation of B cells by IL–4 vary among similar cells derived from different mammalian species. Thus, while IL–4R mobilizes intracellular calcium stores and elevates cAMP in humn tonsillar β cells (275), it does not elicit these effects in murine B cells (276, 277).

Recombinant IL–4 stimulation of B cells results in the specific phosphorylation of a 42–44-kDa protein on tyrosine in some experiments (276, 278); however, contrary results have been reported (discussed in 277). Clearly the molecular events mediating signal transduction from the IL–4 receptor require further characterization to reconcile these disparate observations (277).

IL–3 and IL–5 Receptors

Both the IL–3 receptor and the IL–5 receptor are composed of $\alpha\beta$ heterodimers which share a common β chain with the GM-CSF receptor (279–281). Unlike the β chains of IL–4R and IL–2R, the α chain lacks a serine-rich region (280). Stimulation of growth factor–dependent cell lines with IL–3 reportedly stimulates the translocation of protein kinase C from the cytosol to the membrane, but the evidence is somewhat controversial, and there is no evidence that IP3 is generated or that calcium is mobilized (discussed in 282). However, in hematopoietic cell lines, receptor engagement provokes the phosphorylation of serine and threonine on a number of proteins, including *raf* kinases. IL–3 treatment of IL–3–dependent cell lines also induces the phosphorylation of a series of proteins on tyrosine (77; see 282 for a detailed description) and the activation of $p21^{ras}$ (283–285). An association between the IL–3 receptor and tyrosine kinases has been proposed as a consequence of the observation that a number of oncogenic tyrosine kinases can abrogate the dependence on IL–3. Among such kinases are temperature-sensitive mutants of the v-*abl*-encoded kinase which confer a corresponding temperature-sensitive IL–3 independence upon IL–3–dependent cell lines (286). The significance of this effect is unclear, however, because expression of retroviral protein tyrosine kinases leads to extensive and promiscuous tyrosine phosphorylation. Thus, the identity of the kinase responsible for signal transduction from the IL–3 receptor remains elusive.

IL–7 Receptor

Engagement of the IL–7 receptor, which is normally expressed on pre-B cells, thymocytes, and macrophages (287), leads to the phosphorylation of tyrosines on a number of proteins. In human thymocytes and mature T cells, these events occur without mobilization of intracellular calcium stores (288). In contrast, stimulation of fetal human thymocytes, of an acute lymphoblastic leukemia cell line, and of an immature B cell precursor cell line with rhIL7 results in a rapid stimulation of phospholipid turnover which is inhibitable by the tyrosine kinase inhibitor genistein (289–291). In all cases, the responsible tyrosine kinase(s) remains unidentified.

PHOSPHATASES IN LYMPHOCYTES

The demonstrated importance of protein phosphorylation during the initial stages of lymphocyte activation suggests critical roles not only for protein kinases, but also for phosphatases. The dynamic interplay between these two activities determines the state of phosphorylation of protein substrates that in turn regulate lymphocyte metabolism. Several lines of evidence implicate two distinct phosphatases in the activation of T cells. The first, the leukocyte common antigen or CD45, is a transmembrane tyrosine-specific phosphatase found on the surfaces of all cells of hematopoietic origin (292). Studies involving antibody cross-linking, receptor association, and mutant cells provide evidence for one or more critical roles for CD45 in coupling the engagement of receptors to early activation events. A second phosphatase shown to be important in T cell activation is calcineurin, a calcium- and calmodulin-dependent serine/threonine phosphatase (293). This enzyme has been identified as a target of the immunosuppressive drugs cyclosporin A (CsA) and FK 506 when coupled to their cellular receptors (294), suggesting a potential role in distal activation events.

Phosphotyrosine Phosphatases

CD45 CD45 is a transmembrane glycoprotein which displays a variety of isoforms generated by alternative splicing of the exons encoding a portion of the extracellular domain (292). The cytoplasmic portion, shared by all isoforms, consists of two tandem domains that are strikingly similar to a soluble human placental phosphotyrosine phosphatase (PTPase) (295). This portion of the molecule possesses intrinsic PTPase activity (296). Several lines of evidence indicate that CD45 may regulate receptor-mediated activation in lymphocytes. First, antibody cross-linking of CD45 with specific cell surface receptors inhibits activation. The increased cytoplasmic free calcium concentrations and the subsequent proliferation induced by antibody-mediated cross-linking of CD3 or CD2 on human peripheral leukocytes are blocked when either of these molecules are coaggregated with antibodies to CD45 (297, 298). That this inhibition of calcium mobilization is partially blocked by the addition of vanadate, a PTPase inhibitor, provides indirect evidence for the involvement of the PTPase activity of CD45 in these effects (298). In addition, the tyrosine phosphorylation of a 100-kDa protein and the generation of inositol phosphates initiated by anti-CD2 cross-linking were inhibited by coaggregation with CD45 (299). In mouse T cells, the mobilization of calcium and the increased production of inositol phosphate production, but not their proliferation, were inhibited by coaggregation of CD45 and CD3 (300).

Thus, the physical proximity of CD45 to signaling molecules can inhibit receptor mediated activation events. The demonstration of an association of CD45 with the TCR, CD4 co-receptors, and CD2 accessory structures (301–303) is structural evidence for the involvement of CD45 in regulating signal transduction. However, these experiments suffer from technical limitations imposed by the extraordinarily high surface density (>300,000 molecules/cell in many cases) of CD45 (292).

More direct evidence for an essential role for CD45 was provided by the analysis of T cell clones or transformed lines that lack CD45 expression. Mutant $CD45^-$ T cell clones proliferated in response to antigen or anti-CD3 at only 10% of the response of parental $CD45^+$ cells (304). A $CD45^+$ revertant regained the ability to respond normally to antigen or anti-CD23 stimulation. Similarly, a $CD45^-$ variant of the human T cell leukemic line HPB-ALL failed to mobilize calcium or to generate increased phosphoinositides after stimulation with anti-CD3 (305). Transfection of these variants with a murine CD45 cDNA restored their capacity to mobilize calcium upon triggering of the TCR. The failure of a murine plasmacytoma to mobilize calcium in response to cross-linking of surface IgM was corrected by transfection with a CD45 cDNA (306), indicating a role for CD45 in the signaling of B cells also. Finally, a $CD45^-$ mutant of the human T cell leukemia line Jurkat was shown to be deficient in generating increased levels of phosphotyrosine-containing proteins or IL–2 in response to either anti-CD3 or anti-CD2 (307). These data provide compelling evidence that CD45, presumably via its intrinsic PTPase activity, is essential for coupling the TCR and other receptors to proximal signal transduction pathways including the activation of protein kinases and phospholipase C.

Because the proximity of CD45 to surface receptors inhibits signal transduction, whereas CD45-deficient cells fail to initiate proximal activation events upon triggering of the same receptors, CD45 may well regulate distinct (perhaps even opposing) signaling pathways. This possibility gains support from additional observations on the effects of CD45 on the TCR-associated *src* family protein-tyrosine kinases, $p56^{lck}$ and $p59^{fyn}$. CD45-deficient cell lines show increased phosphorylation of $p56^{lck}$ on tyrosine–505, the putative negative regulatory site (308), suggesting that CD45 PTPase may regulate the kinase activity of $p56^{lck}$ in vivo. Similarly, addition of CD45 in vitro to either $p56^{lck}$ (309, 310) or $p59^{fyn}$ (311) results in rapid dephosphorylation of the regulatory tyrosine and a concomitant increase in kinase activity. In contrast, coaggregation of CD4 and CD45 inhibits the stimulatory phosphorylation and activation of $p56^{lck}$ induced in vivo by anti-CD4 cross-linking (312). Although $p56^{lck}$ and $p59^{fyn}$

appear to be substrates of CD45 PTPase, the outcome of the interaction must depend on the nature of the complex formed.

Therefore while CD45 performs a critical function in coupling surface receptors to signal transduction pathways, the specific targets of CD45 PTPase activity and the mechanism(s) that lead to inhibition or activation remain unclear. Relevant in vivo substrates have not been clearly identified, although it is likely that CD45 acts on $p56^{lck}$ and $p59^{fyn}$. The inhibitory effect of CD45 coaggregation may reflect either competition with these kinases for critical substrates, thereby uncoupling the receptor from transducing molecules, or alteration of the activity of the kinases themselves. The presence of multiple CD45 isoforms, expressed in a developmentally regulated fashion argues that CD45 may participate in a variety of cell-surface interactions and thereby regulate many intracellular effects (302). Identification of appropriate CD45 ligands will provide insight into the regulation of lymphocyte activation. Furthermore, the reconstitution of CD45-deficient cell lines with various mutant forms of CD45 should provide a direct test of the role of PTPase activity and ligand binding in these regulatory roles. It should be noted that other soluble and transmembrane PTPases exist, which are expressed in lymphocytes (313).

Calcineurin: A Phosphoserine Phosphatase Regulator

Although lymphocytes express a variety of phosphoserine/phosphothreonine-specific phosphatases (314), recent findings on the action of the immunosuppressive drugs cyclosporin A (CsA) and FK506 have led to the identification of the serine/threonine phosphatase calcineurin as a nodal point in signal transduction. CsA and FK506 form complexes with the cellular immunophilins cyclophilin and FKBP, respectively (315). These complexes interfere with certain calcium-dependent T-cell activation events, including the induction of IL–2 gene expression (316). One potential cellular target on which the drugs might act was revealed when cyclophilin-CsA and FKBP-FK506 complexes were found to bind and to inhibit calcineurin phosphatase activity in vitro (168). Calcineurin is expressed at low levels in T cells (317), and its phosphatase activity is inhibited in Jurkat cells by treatment with CsA or FK506 (318). The similar drug sensitivities for inhibition of phosphatase activity and IL–2 production suggest a direct relationship. This hypothesis is strengthened by the observation that overexpression of calcineurin or its catalytic subunit in Jurkat cells increases their resistance to the immunosuppressants and augments their expression of an IL–2 promoter–dependent reporter (319, 320). One model for calcineurin action suggests that the phosphatase alters IL–2 expression by regulating, either directly or indirectly, the

nuclear translocation of the cytoplasmic component of NF-AT (321, 322). Identification of this and other proteins as relevant substrates of calcineurin will facilitate a reconstruction of the pathway from early biochemical changes at the cell membrane to changes in transcriptional activity at target genes.

STRATEGIES FOR THE ELUCIDATION OF LYMPHOCYTE REGULATORY PATHWAYS

As the foregoing discussion makes plain, evidence exists to support a role for protein phosphorylation in the control of all aspects of lymphocyte behavior. Figure 2 presents a schematic summary of T cell signaling pathways that illustrates the enormous complexity of these regulatory circuits, even when viewed at low power. TCR signaling requires CD45 activity and improves in the presence of input from CD4/CD8 co-receptor structures. Each of these membrane-spanning polypeptides has an associated catalytic moiety that regulates protein phosphorylation. For CD45, covalently linked PTPase domains influence the extent of phosphorylation of intracellular kinases and/or their substrates. The CD4/CD8 co-receptors interact with $p56^{lck}$ kinase proteins, and the TCR/CD3 complex itself interacts with both $p59^{fyn}$ and with the ZAP70 protein which is itself almost certainly a protein kinase (242, 243). The CD45, CD4/CD8-$p56^{lck}$ and TCR/CD3-$p59^{fyn}$ signaling elements provide unambiguous tethering points for the elaboration of complex regulatory circuits. In each of these cases, both physical and genetic data demonstrate the importance of the link between an enzymatic function and a transmembrane receptor. We can consider these transmembrane signaling complexes as the first tier of a response hierarchy, acting on a second group of less well characterized elements.

How can the identities of downstream regulatory molecules be defined? Figure 2 highlights those structures for which the biochemical evidence supports an influence of tyrosine phosphorylation on enzymatic function. The kinase that actually modifies PLCγ1, the *ras*-GAP protein, or the PI–3 kinase, as examples, remains a matter of conjecture, and indeed, the significance of these phosphorylations has not been unambiguously demonstrated. Nevertheless, these enzymes and others like them provide a crucial link to the cell surface, summing inputs from multiple distinct membrane-spanning elements. The regulatory scheme outlined in Figure 2, already speculative at the second tier, arborizes at the third tier to include control circuits for which only inferential evidence exists. For example, few doubt that protein kinase C activity crucially affects TCR-induced cell activation, but through what mechanism?

Three novel lines of inquiry may provide insight into the biochemical connections between kinases and their substrates. First, it is already apparent that targeted gene disruption and the use of dominant-negative mutations can provide fundamental new information about lymphocyte signaling pathways (30, 35, 36). Though gene targeting may provide a source of cells with genetically determined signal transduction abnormalities, development of novel, high throughput technologies will be required to permit evaluation of even a fraction of the signaling molecules shown in Figure 2.

A related approach will make use of increasingly detailed knowledge of human genetics. In developed countries, individuals with congenital immunodeficiency diseases are frequently identified, and at least some of these immune abnormalities arise from heritable defects in lymphocyte signaling (323). Careful analysis of rare mutations may often provide insight into more general mechanisms of regulation, as exemplified by the rich harvest of command and control genes adduced through the study of rare heritable colonic malignancies (324). Human primary immunodeficiency diseases will provide raw materials for the identification of lymphocyte signaling pathways of indisputable importance.

Finally, it is not unreasonable to expect that mechanisms may have evolved in certain infectious agents to influence lymphocyte regulatory pathways. Such mechanisms may frequently include secreted toxins. For example, the exotoxin elaborated by *Bordetella pertussis* irreversibly inactivates G_i signaling proteins and thereby alters lymphocyte trafficking (325). Analysis of the effects of this toxin has illuminated mechanisms that control T cell development. More generally, lymphotropic infections that promote or inhibit lymphocyte activation can be viewed as surrogate pharmacologic tools for dissecting control mechanisms. For example, the formation of syncytia associated with HIV envelope glycoprotein expression in human $CD4^+$ T cells is both accompanied by accumulation of phosphotyrosine-containing proteins, and antagonized by protein tyrosine kinase inhibitors (326). The discovery of a phosphotyrosine phosphatase (which in this case presumably alters signaling within infected phagocytes) expressed on the surfaces of certain strains of *Yersinia* (327) demonstrates the subtle and highly evolved nature of these parasitic adaptations. An especially enigmatic example obtains in the case of *Theileria parva*, a protozoan parasite that produces a fatal bovine lymphoproliferative disease (East Coast fever). This pathogen somehow stimulates autonomous IL–2 production by redirecting intracellular signaling mechanisms (328). Application of conventional biochemical and genetic methods to the study of these infection-induced signaling disturbances should permit research efforts to focus on critical control elements—regulatory circuits proved to

be important by the persistance of the pathogen over evolutionary time. Such analyses offer the promise of defining novel pathways that could serve as targets for pharmacologically induced attenuation of autoimmune reactivity.

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ORIGIN OF MURINE B CELL LINEAGES

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Abstract

Until recently, the hematopoietic stem cells (HSC) that appear early in ontogeny were thought to constitute a homogeneous, self-replenishing population whose developmental potential remains constant throughout the life of the animal. Studies reviewed here, however, demonstrated clear differences in the developmental potential of fetal and adult progenitor populations (including FACS-sorted HSC). These studies, which chart the ability of various progenitor sources to reconstitute functionally distinct B cell populations, define three B cell lineages: B–1a cells (CD5 B cells), derived from progenitors that are present in fetal omentum and fetal liver but are largely absent from adult bone marrow; B–1b cells ("sister" population), derived from progenitors that are present in fetal omentum, fetal liver, and also in adult bone marrow; and conventional B cells, whose progenitors are missing from fetal omentum but are found in fetal liver and adult bone marrow. B–1a and B–1b cells share many properties, including self-replenishment and feedback regulation of development. These B cell studies, in conjunction with evidence for a similar developmental switch for T cells and erythrocytes, suggest that evolution has created a "layered" immune system in which successive progenitors (HSC) reach predominance during development and give rise to differentiated cells (B, T, etc) responsible for progressively more complex immune functions.

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INTRODUCTION

B cell populations are distinguishable by an ensemble of properties. No single characteristic distinguishes any population; however, sets of characteristics allow the clear recognition of several populations of cells, all of which share a commitment to the production of immunoglobulin. These populations can be recognized on the basis of differentiation status (e.g. pre-B cells, resting B cells, plasma cells), anatomical localization (e.g. marginal zone B cells, follicular B cells, peritoneal B cell), surface phenotype (e.g. IgM and IgD levels, presence of CD5, presence of MAC–1) or, our focus here, the progenitors from which they arise and hence the developmental lineage to which they belong (e.g. B–1 cells formerly known as Ly–1 B cells (1), conventional B cells).

Over the years, several B cell lineages have been proposed. Wortis suggested that the B cells in nude mice and the B cells found in xid mice belong to different developmental lineages (2), and MacLennan and colleagues proposed that splenic marginal zone B cells are a distinct lineage (3, 4). Recently, Linton et al described two precursor populations in the spleen that mostly yield either primary antibody forming cells (AFC) ($J11D^{hi}$) or secondary AFC ($J11D^{lo}$) (5). Finally, our laboratory and others have identified a population of $CD5^+$ B cells in the peritoneal cavity that several laboratories have now collectively shown to belong to a distinct developmental lineage. In this review, we focus on the evidence underlying this major lineage distinction (i.e. B–1 vs conventional B cells) and the substantial evidence for distinct progenitors that has accrued.

Definition of a Developmental Lineage

Webster's Dictionary defines lineage as "descent in a line from a common progenitor" (6). Developmental biologists adhere to this definition; however, there is often considerable discussion, particularly with respect to the immune system, as to what characteristics define a lineage and its progenitor. This definition is often made on practical grounds: in the broadest sense, all cells in a given animal can be assigned to a single lineage, since the zygote is the ultimate progenitor; at the other extreme, the progeny of a single, newly arisen B cell can be treated as a lineage because such B cells are distinguished from each other by unique immunoglobulin rearrangements. By and large, however, developmental lineages are defined as deriving from relatively undifferentiated progenitors that have at least a limited capacity for self-renewal, and they give rise to progeny that are committed to differentiate into cells with particular functional characteristics.

Originally, a single hematopoietic stem cell (HSC) was thought to be

the progenitor of all cells in the hematopoietic system. This stem cell was recognized in early fetal tissue and in adult bone marrow and spleen by its ability to reconstitute an apparently normal hematopoietic system in irradiated recipients (for review, see 7–9). In essence, viewed with the methodology available at the time, the lymphoid, erythroid, and myeloid cells regenerated from either the fetal or the adult HSC appeared identical. Thus, the HSC was assumed to perpetuate itself without change.

Evidence potentially inconsistent with this view has begun to accumulate. For example, studies of erythroid differentiation in the sheep indicate that the early fetal HSC are committed to giving rise to fetal erythrocytes, which express $\alpha_2\gamma_2$ hemoglobin, whereas more mature HSC are committed to generating erythrocytes of the adult phenotype ($\alpha_2\beta_2$ hemoglobin) (10–13). More recently, similar reconstitution studies showed that HSC in adult bone marrow fail to regenerate murine $CD5^+$ B cells which were newly identified by multiparameter FACS analyses (14). Nevertheless, the idea that fetal HSC perpetuate themselves without changing throughout adulthood dominated immunological thinking until recently, when compelling evidence demonstrated differences between the reconstitution potential of fetal and adult HSC.

Data showing that progenitors found at different times during ontogeny are committed (programmed) to differentiate into particular lymphocyte populations come from separate studies of B and T cell development. This review focuses on the B cell studies, which raised the initial challenge to the "single progenitor" hypothesis for lymphocytes and which have now provided definitive evidence for distinctive progenitors for B cell subsets and hence for distinctive B cell lineages. However, similar arguments can be made for T cells. For example, Ikuta and coworkers have dramatically demonstrated that Vγ3 T cells, which are the first T cells to develop in the thymus, (15–17) can develop from fetal HSC but not adult bone marrow HSC (6, 18). Taken together, these findings concerning the origins of lymphocyte subsets force the enlargement of the older paradigm to allow for changes in the potential of the HSC that function at different times during development.

DISTINGUISHING B CELL SUBSETS

In this section, we summarize the properties of the B cell lineages. Because we are primarily interested here in the ontogeny of these lineages, we focus mainly on those whose origins have been extensively investigated. Thus, we treat "conventional" B cells (which include almost all of the B cells in lymph node and spleen) as a single entity even though subdivisions that may reflect additional lineage distinctions have been described. In contrast,

although the B–1 cell population (1), which is concentrated in extralymphoid sites such as the peritoneal and pleural cavities, is substantially smaller, we subdivide it into two separate populations, B–1a cells ($CD5^+$ or $Ly\text{–}1^+$ B cells) and B–1b cells ($CD5^-$ Ly–1 B sister population). This subdivision is consistent with evidence (discussed later) indicating that these two quite similar cell types arise from separate progenitors and thus represent separate lineages. Several other reviews that focus on the phenotype, repertoire, and functions of B–1 cells, including the homologous human population, are available (19–26).

Cell Surface Phenotype

The introduction of multiparameter FACS analysis has facilitated the characterization of B cell populations on the basis of cell surface antigen expression and size (27). This method led to the identification of murine B cell tumors that express Ly–1 (CD5), a cell surface glycoprotein which previously had been thought to exist only on T cells (28, 29). Subsequently, a subpopulation of splenic B cells that express IgM and IgD was shown also to express low to moderate levels of Ly–1 (30–32). Other markers have been used to further characterize the differences between the Ly–1 B cells, which we now call B–1a cells (1): conventional B cells, also known as B–2 cells, and other B cell populations. Peritoneal B cell populations are identified by FACS in Figure 1, and Table 1 summarizes some of the differences in cell surface phenotype for B–1 and conventional B cells.

Two markers deserve special attention. Mac 1(CD11b) is present on peritoneal and pleural cavity B–1 cells but is not expressed on either conventional B cells or splenic B–1 cells. FcεR (CD23) is present on all conventional B cells in the peritoneal cavity and on the predominant

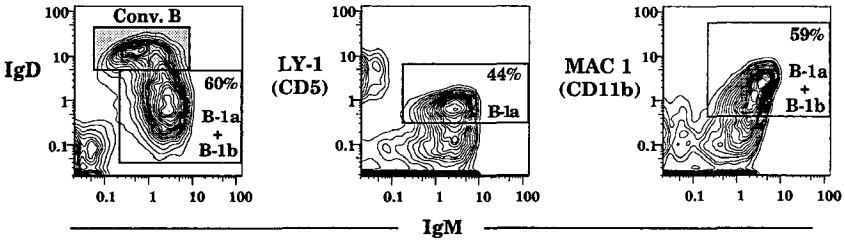

Figure 1 FACS analysis of peritoneal B cell populations. Conventional B cells are identified by a broad, positive IgM and tight, bright IgD FACS profile. They are negative for CD5 (Ly-1) and Mac 1. All B–1 cells are IgM bright and low to moderate for IgD. B–1 cells are also MAC 1 positive in the peritoneum. B–1 cells are divided into B–1a cells which are $CD5^+$ and B–1b cells which are $CD5^-$. The number of B–1b cells is obtained from the difference of total B–1 cells and B–1a cells. Direct gating on $CD5^-$ IgM^{hi} cells is avoided because of overlap with conventional B cells. Plots are 5% probability contours, generated with gating for live lymphocytes by forward and obtuse scatter and propidium iodide.

Table 1 Selected markers on B-1 and conventional B cells. References (27, 30, 31, 33–35, 41, 43, 154), references therein and unpublished observations. Here, conventional B cells do not include marginal zone B cell which are IgD^{lo} and $Fc\varepsilon R^-$

Marker	B-1 cells	Conventional B cells
IgM	+++	+
IgD	+/− to ++	+++
CD5 (Ly-1)	+ on B-1a, − on B-1b	−
CD11b (MAC 1)	+ in PerC, − in Spleen	−
CD23 (FcεR)	−	+
B220 (RA3-6B2)	++	+++
B220 (other)	+	+
IL5R	+	some, inducible
CD72	+	++

(IgD^{hi}) conventional B cell population in the spleen; however, it is not expressed on either marginal zone (IgD^{lo}) B cells in the spleen or B–1 cells from any location. Thus, in the peritoneal cavity (but not in the spleen), these markers alone can be used to distinguish B–1 cells from conventional B cells, i.e. B–1 cells are $Mac1^+$ and $Fc\varepsilon R^-$ whereas conventional B cells are $Mac1^-$ and $Fc\varepsilon R^+$ (33–35).

ACTIVATION MARKERS Several other markers can be used to distinguish B–1 cells from the typical "resting" conventional B cells (IgD^{hi} $Fc\varepsilon R^+$) that predominate in spleen and lymph node. Some of these, however, are also expressed on some types of "activated" conventional B cells. This has raised questions about the activation status of the B–1 population.

The definition of an activated B cell is necessarily vague since the activation protocols can engender different differentiation states that express overlapping but distinct subsets of markers. For example, there is no difference in the level of IL–2R or transferrin receptor expression, presumed markers of intermediate B cell activation, on $CD23^+$ (conventional B cells) and $CD23^-$ (marginal zone + B–1 cells) splenic B cells (36). The majority of both populations are also negative for S7 (CD43), a marker reported to be present on B cells undergoing terminal B cell differentiation (36, 37). However, further studies with S7 reveal that many splenic and peritoneal B–1 cells express the marker, including those cells that secrete antibody (S. M. Wells, A. B. Kantor, A. M. Stall, in preparation). LPS stimulation, which in vivo leads readily to IgM secretion and the development of IgM-secreting plasma cells, induces BLA–1 and BLA–2 expression, but not CD5 on splenic B cells (38). In contrast, Yingzi et al have shown that CD5, as well as some other markers associated with the B–1a phenotype, can be induced on splenic conventional B cells

following in vitro stimulation with mitogenic anti-μ plus IL–6 (39). The authors suggest that their in vitro stimulation with anti-μ is a model for in vivo T-independent (type 2, TI–2) stimulation which produces B–1a-phenotype cells from conventional (IgD^{hi}) B cells.

However, the B–1a phenotype is not induced with the classic TI–2 antigen, TNP-Ficoll. Hayakawa et al found that the plaque forming cells (PFC) are *not* in the FACS sorted Ly–1 B cell population; <2% of the TNP-PFC were $Ly\text{–}1^+$ B cells (105). Anti-bromelein-treated mouse red blood cell (BrMRBC) (105) and antiphosphorylcholine (PC, T15 idiotype) PFC (39a, 115) are found in the $Ly\text{–}1^+$ B cell fraction, indicating Ly–1 is indeed retained on B–1a PFC.

Also, contrary to the results of Ying-zi et al, stimulation of splenic B cells with multivalent anti-μ or anti-δ coupled to dextran (both of which are potent TI–2-like antigens and which extensively cross-link sIg and cause proliferation—40) does not induce CD5 expression. Moreover, the IL–5R, which is found on B–1 cells (41, 42), is induced on splenic conventional B cells with the anti-Ig-Dex stimulation (43). The difference between the two protocols may relate to the specific MAbs used and/or the level of endotoxin contamination in the preparation. Thus, there is still much to be learned about the significance of the expression of various "activation" markers and how they relate to the activation state(s) of the cells they mark.

CD5 EXPRESSION: B–1a VS B–1b CELLS Murine B–1 cells were initially identified because they had low but clearly detectable levels of surface CD5. Later, as FACS technology improved and the characteristic phenotype of these cells became more clearly delineated, we recognized a $CD5^-$ B–1 cell subpopulation whose phenotype, localization, functionality, and replenishment characteristics appear to be identical to the $CD5^+$ B–1 cells (33, 34, 44). Reconstitution studies discussed later (see Progenitors) suggest that these two very similar subpopulations represent closely related but distinct B cell lineages that are reconstituted by separate progenitors. We refer to the cells in these populations/lineages as B–1a cells, which do express detectable levels of surface CD5, and B–1b cells, which do not.

The distinction between the B–1a and B–1b lineages is also reflected in the genetically controlled variation in their frequencies in different mouse strains. Thus, there is genetic variation in the number of B–1a cells (21, 29) and in the number of B–1b cells (34) found in different mouse stains. For example, the fraction of PerC B–1 cells that are B–1b is 20–25% in Balb/c congenics and 40–50% in CBA congenics. The RIIS/J strain is reported to have low levels of peritoneal B–1a cells, but many B–1b cells (45, 46). Thus far there are no known functional differences between B–

1a and B–1b cells. However, the absence of CD5 on B–1b cells and the presence of its ligand CD72 (47, 48) on both B–1a and B–1b cells suggest that such differences will be found.

FACS DETECTION OF B–1 CELLS In principle, the recognition of B–1 cells should be readily achievable in all laboratories that have adequate FACS instruments that are maintained in good condition. In practice, however, certain precautions must be observed (for general reviews, see 49, 50). First, the machine should be standardized before each use, preferably with stable dye-encapsulated polystyrene microspheres, to ensure reproducibility. Second, care should be given to the reagents used: they should be appropriately specific, bright, and titrated to ensure saturating levels without unnecessary background. For example, in the type of B cell transfers discussed here, anti-Ig allotype reagents must not cross-react with the other allotype, and the anti-CD5 reagent must be bright enough to distinguish B–1a and B–1b cells from each other and from T cells. Third, fluorescence compensation for dye overlap should be set properly.

Fourth, for the detection of rare cells, background staining should be minimized and doublets excluded by appropriate counter staining and gating. For example, a doublet containing a $CD5^+$ T cell and an IgM^+ B cell, which might be counted as a B–1a cell in the evaluation of thymic T cells, could be avoided by excluding T cells with CD4 and CD8. Dead cells should also be gated out with propidium iodide. Finally, it is advantageous to evaluate particular B cell subsets with multiple markers. For routine analysis of peritoneal B cells we always use IgM, IgD, CD5, and Mac 1, and often use CD23 and B220 (RA3–6B2).

Anatomical Localization

B–1 and conventional B cells can be distinguished by their anatomical localization. B–1 cells develop early in ontogeny and are readily detected in the neonatal spleen (31). In the adult, B–1 cells predominate in the peritoneal and pleural cavities (14, 51, 52) but are rare in lymph node, Peyer's Patches, and peripheral blood. B–1 cells represent a few percent of the total B cells in adult spleen, most of which are conventional B cells. Note, however, that there is an approximately equal number of B–1 cells in the spleen and peritoneum, e.g. $\sim 3 \times 10^6$ in a normal BALB/c adult. The small number of Ig^+ B cells detected in thymus cell suspensions has also been reported to be $CD5^+$ (53).

Progeny of B–1 cells are also clearly detectable. B–1 cells give rise to large numbers of Ig-secreting plasma cells. Although there is no known distinguishing phenotype for these cells, they can be identified in Ig-allotype chimeras with appropriately allotype-specific reagents. In particular,

the B–1 population makes a large contribution to the IgA-secreting plasma cells of the intestinal lamina propria and the IgM-secreting cells in the spleen (54–56).

B–1 Cells Are Self-Replenishing

In contrast to conventional B cells, which are replenished throughout life by differentiation of unrearranged progenitors based in the bone marrow, B–1 cells maintain their numbers in adult animals by self-replenishment (14, 57). Both kinds of B cells turn over at the same rate (1% day^{-1}) (58–60). However, virtually no newly differentiated B–1 cells enter the peripheral pool in adults, whereas undifferentiated progenitors in the bone marrow continually give rise to (newly arisen) conventional B cells.

In vivo labeling studies have shown that although bone marrow directly gives rise to splenic B cells, few ($\sim$1%) of these newly formed B cells enter the long-term recirculating pool (61). These data mainly reflect the dynamics of the conventional B cell population. Adult bone marrow contains few if any self-replenishing B–1 cells and largely fails to reconstitute B–1 cells (particularly B–1a cells) when it is transferred to irradiated recipients (14, 62, 63). Thus it mainly provides a continuing progenitor source for the replenishment of conventional B cells.

B–1 cells, in fact, neither need nor use a continuing progenitor source in adults. Reconstitution studies show clearly that B–1 cells can maintain their numbers by self-replenishment, i.e. by division of fully mature B–1 cells. FACS-sorted IgM^+, $CD5^+$ B–1a cells completely and permanently reconstitute the B–1a population in transfer recipients (57). Similarly, FACS-sorted B–1b cells completely and permanently reconstitute the B–1b population. Within experimental limits, each sorted B–1 population replenishes itself but not the other (33, 44), indicating that these populations are independently maintained. In addition, in vivo feedback regulation studies (see Feedback) show that the entry of cells into the B–1 pool terminates shortly after weaning, since depletion of a component of the neonatal B–1 population results in the depletion of that component throughout life. Thus, the in situ B–1 population must persist via self-replenishment rather than de novo differentiation.

CAN CONVENTIONAL B CELLS RECONSTITUTE THEMSELVES? The question of whether some conventional B cells also persist via self-replenishment is more difficult to address. Antigenic stimulation induces IgM^+, IgD^+ conventional B cells to differentiate into memory B cells that mainly switch to IgG-expressing cells that persist for the life of the animal. These cells, which may divide infrequently in situ in the absence of antigen, readily reconstitute the memory population in (antigen-stimulated) adoptive

recipients and thus qualify as self-replenishing cells (64). They do not, however, reflect the behavior of typical IgM-bearing conventional B cells, which consistently fail to reconstitute the overall conventional B cell population when FACS-sorted cells are transferred to irradiated recipients.

Small numbers of transferred conventional B cells may persist for many months in adoptive recipients and may even be capable of limited self-replenishment. Careful analysis reveals their presence in appropriate recipients (65) (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). Their low numbers suggest either that they persist without being able to divide at a rate sufficient to replenish the overall conventional B cell population, or that they represent a unique subset of self-replenishing conventional B cells.

Because the issue of the persistence of these transferred conventional B cells has raised questions concerning the difference in potential for self-replenishment between conventional and B–1 cells (65), it is important to consider the experimental detail underlying the above conclusions. In effect, transferring $1–2 \times 10^6$ peritoneal or splenic B–1 cells (with supporting bone marrow) to irradiated recipients results in the essentially complete and permanent reconstitution of the B–1 population in the peritoneal cavity and apparently at all other sites to which B–1 cells migrate (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). Conservative estimates indicate that there are roughly $7–10 \times 10^6$ B–1 cells in the adult BALB/c mouse and thus that the transferred B–1 cells must have increased their numbers by at least 3–5 fold. Since these estimates take into account the number of B–1 cells in the spleen and the peritoneal and pleural cavities but do not include the number of plasma (or other) cells derived from B–1 cells in sites like the spleen and gut, it is likely that the transferred B–1 population expands considerably more than we estimate.

In contrast, data from transfers of conventional B cells indicate that roughly half the injected B cells are recoverable in the recipient several months after transfer (these do not include plasma cells, etc). For example, Sprent and colleagues report the presence of roughly $3–5 \times 10^6$ donor B cells in SCID recipients that received 10^7 lymph node B cells (65). Similarly, we estimate that we recover roughly $1–2 \times 10^6$ conventional B cells in BALB/c mice that received either 2×10^6 lymph node B cells or a similar number of FACS-sorted splenic conventional B cells (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). (Note that better Ig-allotype specific detection is required to identify these cells in reconstituted irradiated recipients than in SCID recipients because of the large number of B cells derived from the obligatory cotransferred bone marrow.) Thus while the B–1 cell population expands 3–5 fold in an adoptive recipient,

conventional B cell populations tend to shrink in size. This difference, while not overwhelming like the expansion of transferred stem cells, is an important distinction between B–1 and conventional B cells, particularly in light of evidence indicating that the B–1 population formed in neonates persists for the life of the animal (see Feedback).

Measurement of B cell turnover by the incorporation of BrdU is consistent with the above results. B–1 cells turnover at about 1% per day, based either on measurements of total PerC B cells (59, 60) or histologically identified B–1 cells (58, 58a). Conventional B cells have a similar turnover rate (59, 60). Measurement of peritoneal B cells in $S+G_2/M$ phases of the cell cycle yields figures consistent with these data (66, 67). Reports suggesting much higher values for peritoneal B cells in $S+G_2/M$ phases of the cell cycle ($\sim$20%) (47, 61) may be explained by technical problems, e.g. the failure to exclude doublets from the FACS analysis. Freitas and colleagues, using clever but perhaps riskier systems, also dispute the 1% per day turnover estimate for peripheral B cells (68, 69) as being at least 10-fold too low (68, 69). Further discussion of the many, often conflicting, turnover experiments are beyond the scope of this review.

Feedback Regulation of B–1 Development

The studies discussed above focus on a central question for peripheral B cell dynamics: how important are the processes of self-replenishment and de novo differentiation from progenitors in the "turnover" of B–1 and conventional B cells in intact animals. Answers to this question, unfortunately, are difficult to obtain from cell transfer studies since interpretation of the data requires several key assumptions, e.g. that the "drainage" from the B cell populations into dead cells, plasma cells, etc, is equivalent for conventional B cells and B–1 cells and can therefore be ignored when estimating population expansion.

Results from studies investigating the in situ depletion and recovery of the B cell populations following neonatal treatment with anti-Ig antibodies provide a clear statement on this issue. In essence, Lalor and coworkers in our laboratory have shown that a feedback mechanism that regulates the development of B–1 cells from immature progenitors prevents the emergence of newly differentiated B–1 cells (both B–1a and B–1b) but does not interfere with the development of conventional B cells (44, 70). These findings indicate that B–1 development from undifferentiated progenitors terminates in intact mice somewhere between 3 and 6 weeks of age, whereas conventional B cells continue to develop from immature progenitors throughout the life of the animal.

These studies confirmed earlier findings showing that treatment of neonatal inbred mice with anti-IgM antibodies depletes all B cells, and that

normal numbers of B cells return after the treatment Ab disappears (71). In addition, we showed that this recovery extends to both the B–1 and conventional B cells (when all B cells have been depleted), and that monoclonal antibodies to allotypic determinants on IgM (i.e. anti Igh–6b) can also be used to deplete B cells. Thus, the stage was set for comparing the recovery of B–1 and conventional B cells both in *allotype homozygotes*, where the treatment Ab depletes all B cells, and in *allotype heterzygotes*, where the treatment Ab depletes only half of the B cells (i.e. those that express the reactive IgM allotype).

Studies with both kinds of mice yield essentially the same result: conventional B cells recover to normal frequencies shortly after the treatment Ab disappears; B–1 cells, in contrast, only recover when there are no B–1 cells in the animal. B–1 cells fail to recover in allotype heterozygotes, in which only half the B cells (conventional and B–1) are depleted by the treatment Ab. Similarly, they fail to recover in Ab-treated homozygotes in which mature allotype congenic B–1 cells (or a benign B–1 cell tumor) have been introduced during the neonatal period. The presence of mature B–1 cells is necessary and sufficient to prevent the de novo development of B–1 cells in intact animals.

The block in B–1 development proved to be permanent both in the Ab-treated allotype heterozygotes and in the treated homozygotes in which the B–1 cells were restored. More than 6 months after the treatment antibody disappeared and the depleted conventional B cell population recovered, B–1 cells expressing the reactive IgM allotype remained below detectable levels. Thus, we conclude that the B–1 population that develops during the first few weeks of life in normal animals prevents the subsequent entry of newly arisen B–1 cells into the peripheral pool throughout life.

EVIDENCE FOR DISTINCT B CELL PROGENITORS

The dramatic differences between B–1 and conventional B cells discussed above support the idea that they belong to separately developing lineages. The first actual data indicating that this lineage distinction exists, however, came from early cell transfer studies which demonstrated that adult bone marrow readily reconstitutes conventional B cells but only poorly reconstitutes $CD5^+$ B cells (14). Now, some seven years later, a variety of studies confirm and extend this initial hypothesis, demonstrating that conventional B cells and B–1 cells belong to separate developmental lineages (62, 72–75) , and suggesting a similar lineage split between B–1a and B–1b cells (62). Rather than discussing these lineage studies in their historical order, we have chosen to organize this section to consider data demonstrating (i) that fetal omentum contains progenitors for B–1 cells

but not conventional B cells, (ii) that fetal liver contains progenitors for both lineages, (iii) that progenitors for B–1 cells, particularly B–1a ($CD5^+$) cells, are depleted in adult bone marrow, and (iv) that FACS-isolated pro-B cells from fetal liver and adult bone marrow are committed to develop, respectively, into B–1 and conventional B cells. It should be noted that we use "progenitors" in a broad sense; the term may include cells ranging in potency and commitment from HSC to pro-B cells. When the data warrants, we employ more specific designations.

Progenitors in Fetal Liver and Omentum

FETAL OMENTUM CONTAINS PROGENITORS FOR B–1 CELLS Solvason and colleagues have shown that 13-day fetal omentum reconstitutes B–1a and B–1b cells *but not conventional B cells* when grafted under the kidney capsule of (or suspended and transferred into) SCID mice (75–78). Since omental tissue at this fetal age does not contain Ig^+ cells (77), these findings demonstrate (i) that a distinct site associated with the mesodermal-peritoneal lining houses Ig^- progenitors specifically committed to differentiate to B–1 cells, (ii) that such progenitors exist, and (iii) that these progenitors develop in adoptive hosts according to their original commitment.

The specific progenitors responsible for the B–1 cell reconstitution have not been identified; they could be HSC, lymphoid progenitors, pro-B cells (see Isolation), or a mixture. The fetal omentum also contains progenitors for T cells, demonstrable by cografting fetal omentum with fetal thymus from a genetically distinct donor. This suggests that at least some progenitors in the omentum are not yet committed to the B cell lineage. These findings extend pioneering work by Kubai and Auerbach showing that fetal omentum is a source of lymphocyte progenitors in the mouse (79).

FETAL LIVER CONTAINS PROGENITORS FOR B–1 AND CONVENTIONAL B CELLS Like fetal omentum, fetal liver (13 and 14 day) does not contain Ig^+ B cells (80) and readily reconstitutes both B–1a and B–1b cells. However, unlike fetal omentum, fetal liver also reconstitutes conventional B cells (62, 76). Thus, the reconstitution with this tissue comes closest to restoring the normal B cell population frequencies, since transfers of fetal liver reconstitute B–1b and conventional B cells fully and B–1a cells to about half their normal level (62).

Data from the 13 and 14 day fetal liver transfers are consistent with the existence of either one or two B cell progenitors in fetal liver. That is, fetal liver could either contain a single progenitor capable of reconstituting all B cell lineages, or it could contain multiple progenitors committed to develop into distinct B cell lineages. The data from the omentum transfers argue in favor of the latter hypothesis because the 13 day omental tissue,

which is contiguous with the fetal liver capsule, contains only the progenitors for the B–1 lineage(s). In fact, it is possible that the progenitors for B–1 and conventional B cells are actually anatomically separate in the fetal liver, with the progenitors for conventional cells located in the interior of the liver and the progenitors for B–1 cells associated with the capsule. Resolution of this question, however, requires the development of demanding dissection techniques.

PERITONEAL PROGENITORS FOR B–1 CELLS? Marcos et al have presented preliminary data suggesting that there is an adult source of B–1 cells (B–1a and/or B–1b) associated with the peritoneal cavity, perhaps the adult omentum correlated tissue (81). Repeated washing of the peritoneal cavity leads to a loss of B–1 cells. After this in vivo peritoneopheresis is stopped, B cells are reported to return to the peritoneal cavity, first as $B220^+$, IgM^- "pre-B" cells and then as IgM^+ B–1 cells. If these results are confirmed, they suggest an adult source of B–1 progenitors that might function in the event of extreme B–1 cell depletion.

Progenitors in Bone Marrow

Since the early work by Hayakawa and Hardy, which demonstrated that bone marrow fully reconstitutes conventional B cells but largely fails to reconstitute B–1 cells (particularly B–1a cells) (14), a variety of bone marrow transfer studies aimed at answering more subtle questions about the nature of the B cell progenitors in bone marrow have been completed (34, 62, 63). Two new conclusions can be drawn from this work. First, although the new data show that there is more variation in the low levels of B–1a reconstitution from bone marrow than previously recognized, these findings still clearly confirm the earlier evidence indicating that bone marrow contains very little progenitor activity for B–1a cells. Second, the new data confirm and extend earlier evidence (44) indicating that substantial progenitor activity for B–1b cells is present in adult bone marrow and functions when there are very few B–1a cells in the animal (62).

BONE MARROW CONTAINS PROGENITORS THAT FULLY RECONSTITUTE CONVENTIONAL B CELLS The total number of splenic T cells and conventional B cells routinely returns to normal levels or above in bone marrow recipients. However, while conventional B cells comprise 10–20% of the lymphocytes in normal Balb/c PerC, they represent 50–60% of the PerC lymphocytes in bone marrow recipients (62). This increased frequency of conventional B cells mainly reflects the failure to reconstitute normal numbers of B–1a cells.

BONE MARROW CONTAINS VERY FEW PROGENITORS FOR B–1a CELLS In our hands, the level of peritoneal B–1a ($CD5^+$) cells recovered from adult bone marrow transfers is roughly 5% of the number of B–1a cells in normal (intact) animals. This low level B–1a cell reconstitution could be due wholly or in part to rare (self-replenishing) B–1a cells located in the bone marrow; however, since transfers of $B220^-$ bone marrow cells also result in similar low level B–1a reconstitution (58, 59), it is likely that a low frequency of B–1a progenitors survives into adulthood and is revealed in adoptive recipients.

In different experiments involving both BALB/c and CBA mice tested from 2–8 months after transfer, the number of B–1a cells recovered ranges from $<2\%$ to 15% of normal B–1a levels (62, 63) (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). The high levels of B–1a cells recovered could be accounted for by the occasional presence of B–1a clonal neoplasms in the bone marrow source, because these clones tend to expand extensively in adoptive recipients (82). In addition, these high levels could be due to exceptionally high levels of circulating B–1a cells or to B–1a cells localizing in the bone marrow. However, as indicated above, it is likely that the variation in the number of B–1a cells recovered in bone marrow recipients is largely determined by the number of B–1a progenitors that persist in adults.

Other groups claim to obtain substantially higher B–1a reconstitution from bone marrow. Unfortunately, some of these studies have serious technical flaws (83, 84), and others are too incomplete to fully evaluate (85–87). For example, in striking contrast to other published data (14, 34, 44, 62, 63, 72, 73), one study concludes that bone marrow reconstitutes B–1a cells as well as PerC in irradiated recipients (83). The FACS data in this study, however, were analyzed inappropriately: a and b allotype B cells were not adequately resolved; gates were chosen incorrectly, perhaps in part because of the limitation of the contour program used; and the panel of staining reagents was too limited. Another study, which reports moderate bone marrow reconstitution of B–1a cells (84), has similar technical difficulties. This study reports approximately equal levels of IgM^+, $CD5^+$ cells following bone marrow transfers in both the spleen and peritoneum of the irradiated recipients. This contradicts the well-established finding that B–1a cells localize to the peritoneum after transfer (14, 34, 44, 62, 63, 67, 72, 73).

When examined closely, none of the putative findings in the above studies seriously challenges the argument that bone marrow is a poor source of progenitors for reconstituting B–1a cells. In contrast, well-grounded data repeatedly demonstrate that bone marrow largely fails to

reconstitute B–1a cells (14, 34, 44, 62, 63, 72, 73). Since these studies show that conventional B cells are fully reconstituted in the same bone marrow recipients in which B–1a reconstitution largely fails, we interpret this evidence as indicating that conventional B cells derive from different progenitors than B–1a cells.

BONE MARROW TRANSFERS DISTINGUISH PROGENITORS FOR B–1a AND B–1b CELLS We repeated our earlier transfer studies and more closely defined the kinds and frequencies of B cells reconstituted from adult bone marrow. These studies suggested the division of the B–1 population into two B–1 lineages, now provisionally called B–1a and B–1b.

The first evidence suggesting distinct developmental differences between B–1a and B–1b cells came from feedback regulation studies showing that the B–1 population that recovers following neonatal B cell depletion by anti-IgM antibody treatment (of allotype homozygotes) consists largely of B–1b cells. This evidence suggested that functional progenitors for B–1b cells persist longer into adulthood than progenitors for B–1a cells. These studies also established B–1b cells as a distinct population by showing that FACS-sorted B–1b cells are fully capable of self-replenishment in adoptive recipients, and neither derive from nor give rise to B–1a cells (44, 70).

Data from our recent bone marrow transfer studies confirm the independent progenitor origins of B–1a and B–1b cells (34, 55, 63). In agreement with previous data, these studies show that B–1a cells are very poorly reconstituted by progenitors from this source. In addition, however, they show that B–1b cells are routinely reconstituted in bone marrow recipients and, on average, reach half their normal frequency (40, 58, 84, 30, 59). This does not amount to a large reconstitution of the overall B–1 population, because B–1b cells usually represent less than a quarter of this population in the Balb/c animals used for this study. These bone marrow–derived B–1b cells also replenish themselves when peritoneal cells are transferred into a second set of recipients. (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation).

The reconstitution of B–1b cells was difficult to detect in the earlier studies, largely because these studies used a more limited set of cell surface markers and a more limited FACS instrument (two- rather than three-color) to characterize the B cell populations in transfer recipients. In contrast, current FACS and reagent technology reveals the reconstitution of B–1b cells quite clearly and leaves little doubt that they are reconstituted much more efficiently (per cell transferred) by adult bone marrow than are B–1a cells.

This reconstitution data is consistent with the idea that cells expressing

the B–1b-phenotype are derived from two B cell progenitors—one that is most active early in ontogeny and also produces B–1a cells, and one that is active later in ontogeny and also produces conventional B cells. However, evidence from the feedback inhibition studies makes this dual-progenitor hypothesis less attractive. These studies, which show that the development of all B–1b cells is sensitive to feedback inhibition by mature B–1 cells, make it unlikely that a proportion of B–1b cells are derived from the same progenitors that give rise to conventional B cells. Therefore, we interpret the data from the bone marrow transfer studies, which suggest that functional progenitors for B–1b cells survive longer into adulthood than progenitors for B–1a cells, as indicating that B–1a and B–1b cells are derived from independent progenitors and hence that these cells belong to distinct developmental lineages (Figure 2).

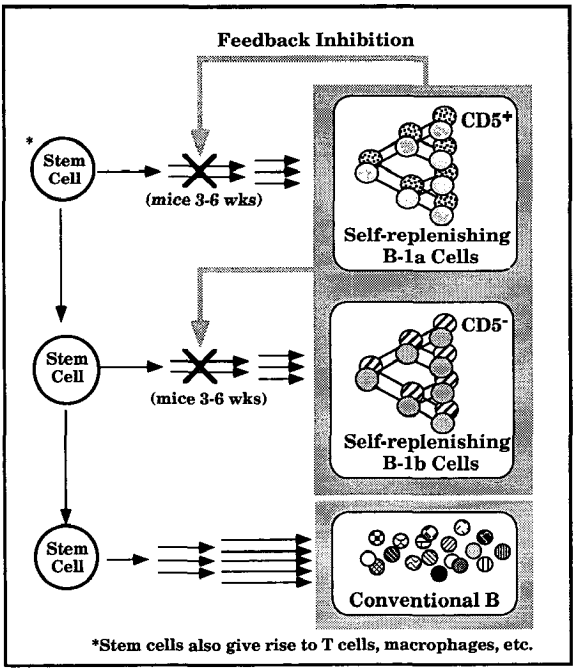

Figure 2 Development and feedback regulation of B cell lineages. The progenitor studies reviewed here demonstrate that B–1a and conventional B cells are distinct lineages. Bone marrow transfers also suggest that B–1b cells constitute a distinct lineage. Feedback inhibition regulates the *de novo* production of both B–1a and B–1b cells.

Cotransfer of Progenitors in Fetal Liver and Adult Bone Marrow

Since B–1a cells readily develop from fetal liver in adoptive transfers, their limited reconstitution from adult bone marrow is not due to conditions inherent in the recipient environment per se. However, the environment in a recipient being reconstituted with fetal liver is not necessarily the same as the environment in a recipient being reconstituted with bone marrow. Fetal-liver derived cells or cell products could be required to support the development of B–1a cells; or, bone-marrow derived cells or cell products could block B–1a cell development. Therefore, before we finally concluded that progenitor activity for B–1a cells is deficient in adult bone marrow (62), we co-transferred fetal liver and adult bone marrow and demonstrated that B–1a progenitors in the co-transferred recipient develop normally and exclusively from the fetal liver source. These studies are described in the next section.

B–1a PROGENITORS ARE DEPLETED IN ADULT BONE MARROW In the studies referred to above, we cotransferred 14-day fetal liver (BAB, Igh b-allotype) and adult bone marrow (Balb/c, a-allotype) into irradiated recipients (62). Analysis of the recipients demonstrated that bone marrow does not contain cells that limit the development of B–1 cells from their progenitors (and hence hide the fetal liver progenitors); and fetal liver does not contain cells that enhance the development of B–1a cells (and therefore reveal cryptic progenitors for these cells. In essence, data from these studies showed (i) that fetal liver and bone marrow reconstitute the same proportion of peritoneal B–1a, B–1b, and conventional B cells whether transferred together or separately; (ii) that bone marrow reconstitutes the B–1b cells better than B–1a cells, as in the separate transfers described above; and (iii) that the ratio of bone marrow–derived B–1b cells to B–1a cells in cotransfer recipients is equal to the ratio observed when bone marrow is transferred alone. Thus, we conclude that bone marrow is clearly deficient for progenitors for B–1a cells.

B–1 AND CONVENTIONAL B CELL PROGENITORS MAY BE DISTINCT IN FETAL LIVER The cotransfer studies discussed above incidentally provided evidence demonstrating that fetal liver transfers can reconstitute B–1 cells without reconstituting conventional B cells. That is, in some cotransfer recipients (3/13), fetal liver readily reconstituted B–1 cells but failed to reconstitute conventional B cells. All conventional B cells in these recipients were derived from the cotransferred bone marrow (62). The simplest explanation for these findings is that the progenitors for B–1 cells and conventional B cells are distinct in fetal liver; and that the progenitors for B–

1 cells were abundant in the fetal liver suspension that was transferred, whereas the progenitors for conventional B cells were rare enough to fail to be expressed in some recipients.

Isolation of B Cell Progenitors

The question of separate progenitors, and hence separate lineages, is best addressed by contrasting the developmental potential of the earliest fetal and adult progenitors in the B cell developmental pathway, i.e. hematopoietic stem cells (HSC) and their committed offspring, particularly pro-B cells. Hardy and Hayakawa have made substantial progress in this area (72–74, 88).

First, Hardy and Hayakawa demonstrated that HSC population(s) from either neonatal liver or adult bone marrow readily reconstitute conventional B cells (in irradiated SCID recipients), whereas B–1 cells are only reconstituted by HSC isolated from neonatal liver. Second, they showed that FACS-sorted pro-B cells from adult bone marrow mainly give rise to conventional B cells whereas pro-B cells from neonatal sources give rise to B–1 cells. This series of progenitor studies, which definitively establishes the independent lineage origins of B–1a and conventional B cells, is summarized in the sections that follow.

RECONSTITUTION WITH FACS-SORTED HSC As expected, when adult bone marrow HSC are enriched by FACS-sorting Thy–1^{lo}/Lin$^-$(B220$^-$, CD4$^-$, CD8$^-$, etc) cells and transferred to SCID recipients, they reconstitute B cell populations similar to unsorted and B220$^-$ adult bone marrow (compare 62, Figure 1, and 72, Figure 1). Notably, conventional B cells are fully reconstituted, B–1b cells are reconstituted at substantial frequencies, and a small but detectable number of B–1a cells are also derived from the donor source. Thus, differentiation from HSC is sufficient to account for the limited reconstitution of B–1 populations from adult bone marrow discussed above.

These transfers also provide evidence for distinct B–1 progenitors in the HSC fraction sorted from neonatal liver. FACS-sorted fetal liver HSC populations contain progenitors for all B cell populations. However, while transfers of 50,000 Thy–1^{lo}/Lin$^-$cells reconstitute both B–1 and conventional B cells, transfers of small numbers (500) of the FACS-sorted HSC only reconstitute conventional B cells (72, 73). These data are consistent with the selective reconstitution of B–1 cells that we observed in several recipients of fetal liver (cotransferred with adult bone marrow). Taken together, these findings add weight to the idea that progenitors for B–1 cells are distinct from progenitors for conventional B cells in fetal liver.

RECONSTITUTION STUDIES WITH FACS-SORTED PRO-B CELLS Hardy and Hayakawa also demonstrated that the difference in B lineage commitment observed for HSC in adult bone marrow and fetal liver is reflected by the commitment of differentiated pro-B cell from these tissues (72–74, 88). They first used multiparameter FACS analysis and sorting to define a differentiation pathway for $B220^+$ pro-B and pre-B cells (88) based on the differential cell surface expression of BP–1 (89), CD43 (leukosialin, S7—37, 90), and heat stable antigen (HSA, 30F1). They defined the differentiation status of the isolated populations with respect to Ig rearrangement: pro-B cells have D_H-J_H but not V_H-D_HJ_H rearrangements, whereas pre-B cells show the full $V_HD_HJ_H$ rearrangements.

Most significantly, sorted adult bone marrow pro-B cells ($B220^+$, $CD43^+$, HSA^+) in the above studies reconstitute mostly conventional B cells, while similarly sorted fetal liver pro-B cells yield only B–1 cells when transferred into lightly irradiated SCID recipients. Thus, pro-B cells are committed to particular lineages when isolated from fetal versus adult sources. The repopulation of conventional B cells from adult bone marrow pro-B cells peaked around 2–3 weeks after transfer and subsequently decreased. The B–1 cells repopulated from fetal liver pro-B cells peaked by 2 months and remained constant thereafter. The repopulation kinetics from fetal and adult pro-B cells are consistent with and provide further evidence of the self-replenishing capabilities of B–1 cells.

The distinct differentiation potential of fetal liver and adult bone marrow pro-B cells was also demonstrated with short-term stromal layer cultures (74). Fetal liver pro-B cells yield mostly $CD5^+$ B cells whereas adult bone marrow pro B cells yield mostly $CD5^-$ B cells. Thus, in vitro results are completely compatible with the results from in vivo studies. The development of the $CD5^+$ (B–1) B cell population in the fetal pro-B cells cultures bears on another question of importance. It demonstrates that the phenotype of B–1 cells is not defined by in vivo influences, e.g. interaction with maternal antibodies or with self-antigens other than those expressed by the restricted set of cells in the stromal culture.

B CELL ANTIBODY REPERTOIRE

Developmental differences may be important in determining functional distinctions among the B cell lineages. Here we review some of the evidence for differences between the repertoires of B–1 and conventional B cells with respect to isotype, specificity, response and rearrangement machinery. We also consider the influence of selection on the expressed repertoire of the individual lineages and possible influences of selection on the phenotype of B cells.

This discussion is necessarily incomplete because current information is not sufficient to draw conclusions on key issues relevant to our focus here, that is, the lineage origins of B cells. For example, although B–1 cells have been clearly shown to predominate in the response to certain antigens, further studies are required to determine whether these functional differences reflect differences in the potential of the lineages to express the particular Ig rearrangements used in these responses. Similarly, although initial studies suggested that V gene representation in the peripheral B–1 cell repertoire is considerably more restricted than in the conventional B cell repertoire, subsequent studies reopen this question by demonstrating substantially more diversity in the B–1 cell repertoire, with respect both to V_H gene representation and to N-region insertion.

Resolution of these issues has been hampered by the lack of adequate methodology to define the native repertoires of the B cell lineages. Much of the early V gene data comes from hybridomas or mitogen-stimulated B cells, which of necessity define selected repertoires dependent on functional response potential. Other data, based on cDNA amplification of sorted or ontologically isolated B cell populations, is skewed toward the most abundant messages in a particular population. Overall, therefore, although considerable data has been amassed, the native (and locally selected) repertoires of the lineages have yet to be clearly defined.

The question of B–1 and conventional B cell participation in T-dependent and T-independent responses also has yet to be fully resolved. B–1 cells are responsible for producing many commonly studied autoantibodies and antibacterial antibodies; however, the assumed extension of this evidence to the idea that B–1 cells only produce T-independent responses is incorrect. Thus, although there are a number of generally accepted ideas about the repertoires of B–1 and conventional B cells, corrections and caveats apply to many of the interpretations given to the data. These and related issues are discussed in the sections that follow.

The B–1 Antibody Responses

IMMUNOGLOBULIN ISOTYPES Although the antibody responses in which B–1 cells have been studied tend mainly to be IgM (e.g. to bromelain treated erythrocytes), B–1 cells can produce all Ig isotypes. They make major contributions to serum IgM, IgG_3, and IgA (33, 76, 91) and produce a large percentage of the IgA-producing plasma cells in the gut (54–56, 92). The B–1 cell contribution to total serum IgM is dramatically demonstrated in mice treated with anti-IL–10 antibody (92a). B–1 cells, which are the main source of B cell–derived IL–10 (92b), are completely depleted from the peritoneum by the anti-IL–10 treatment and serum IgM is drastically

reduced, to $<10\%$ of normal. Conventional B cells, which remain in the treated mice, are still able to make specific IgM in response to TNP-KLH.

Studies with B–1 cell lines indicate that cytokines (e.g. IL–4) regulate the switching of stimulated B–1 cells to the more advanced isotypes (93–95). Since T cells are likely to be the major source of such cytokines in antibody responses, these isotype switch data suggest that the characteristics of B–1 antibody responses are regulated by T cells in much the same manner as the responses of conventional B cells are thought to be. However, the question of affinity maturation and somatic mutation in the B–1 cell–derived IgG and IgA-secreting plasma cells is still unresolved.

CLONAL POPULATIONS OF B–1 CELLS Virtually all mice over the age of 15 months have clonal populations of B–1 cells detectable in Southern gel analyses of splenic or peritoneal lymphocytes (96). These clonal B–1 populations, which are also detectable by FACS analysis when they become large, are present in many older mice (>5 months of age) and can even be found in neonates from some mouse strains (e.g. NZB) (82). They appear quite frequently in irradiated recipients reconstituted with peritoneal B cells from older mice (82) and in nonirradiated neonatal mice injected with peritoneal cells (67). On occasion, spleen and bone marrow can also yield clones (82) (and unpublished observations). Unfortunately, when present, these clonal populations skew the results of repertoire analyses and can lead to erroneous views of the overall B cell repertoire. V gene studies have associated B–1 populations with the expression of a limited, germline repertoire; however, the presence of B–1 clonal populations may have overemphasized the extent of this restriction in some studies (82, 96–99). The repertoire in unmanipulated young mice appears to be more diverse, at least within the J558 family (100).

The influence of feedback regulation and the emergence of clonal populations on the development of the B–1 repertoire are summarized in Table 2. In essence, the B–1 repertoire is fixed early in development and becomes progressively restricted as animals age, because new entrants to the B–1 pool are prevented (due to the feedback mechanism), and clonal populations expand to occupy a progressively greater proportion of the pool.

B–1 ANTIBODY RESPONSES B–1 cells respond well to some multivalent antigens (T independent), especially in connection with the production of auto- and anti-bacterial specificities. They produce the major response to microorganismal coat antigens such as lipopolysaccharide (101) α1–3 dextran (67), phosphorylcholine (PC, T15, idiotypes; 101a) and undefined determinants on *E. coli* (102) and Salmonella (103). In addition, they respond to another bacterial coat component, phosphatidylcholine (PtC), which is often measured as reactivity to bromelain-treated mouse red

Table 2 Repertoire development in B cell lineages

Stage	Age	B-1 cells	Conventional B cells
Fetal	12–13 days	Progenitors (HSC) appear in the liver and omentum	Progenitors appear in the liver (not in omentum)
	>16 days	Progenitors (including pro-B cells) begin to give rise to B-1 cells	
		Selective forces start to shape the repertoire potential	
Postnatal	0–4 weeks	Progenitors continue to give rise to self-replenishing B-1 cells; population approaches adult size	Population starts to enlarge
Adolescent	4–8 weeks	Feedback inhibition blocks new development from progenitors	Population approaches adult levels; de novo differentiation from progenitors continues
		Repertoire potential becomes fixed	
Adult	8–20 weeks	Individual clones expand or are deleted	Population reaches maximal levels (12–14 weeks); de novo differentiation from progenitors continues
		Repertoire becomes progressively more restricted	
Elderly	>20 weeks	Hyperplastic and neoplastic (B-CLL) clones appear	

blood cells (104, 105) or PtC-containing liposomes (106). This reactivity uses V_H11 and V_H12 almost exclusively (107–111a) and accounts for $\sim 10\%$ of the peritoneal B–1 cells. B–1 cells also produce other autoantibodies, e.g. to thymocytes (112).

T dependent vs T-independent responses The ready responsiveness of B–1 cells to bacterial coat antigens and other typical T-independent (TI) antigens appears to have led to the erroneous idea that responsiveness to T-dependent (TD) and TI antigens distinguishes B–1 cells from conventional cells. It is true that B–1 cells do not respond very well to certain laboratory antigens commonly used to study TD antibody responses, e.g. B–1 cells respond poorly to sheep erythrocytes and TNP (22, 105) and NP (67) haptens in TD (protein coupled) form. However, B–1 cells also do not produce a clearly detectable plaquing response to TNP or NP in a TI (Ficoll coupled) form (21, 67, 105). Thus the ability to respond does not hinge simply on the form in which an antigen is presented.

The lack of response to the TNP hapten could reflect the state of the B–1 repertoire and/or an inability to stimulate somatic mutation and affinity maturation in B–1 cells with either the TI or TD form of TNP. In vitro LPS stimulation studies reveal a high frequency of FACS-sorted B–1 cells that produce antibodies that bind to TNP; however, these antibodies are broadly reactive and have a low affinity. Thus, they differ from the relatively high affinity, fine specificity antibodies that are elicited even in a primary TD anti-TNP response produced in vivo by conventional B cells (113).

B–1a cells do, however, produce TD responses to certain antigens. For example, they are the major source of the dominant $T15^+$ idiotype in the antibody response to phosphoryl choline (PC) (101a, 114), which Taki et al have demonstrated is stimulated by the TD antigen PC-KLH (115). Also, A/WySNJ mice, which have B–1 cells but are deficient in conventional B cell development, make good primary IgM responses to both TD and TI antigens, but poor secondary IgG responses (116, 117). Thus, although they are selective with respect to antigen, B–1 cells are capable of making both TD and TI responses.

T cells clearly influence other aspects of antibody production by B–1 cells. Huetz and coworkers described the dependence of the LPS-driven anti-PtC response on CD4 T cells (117a). Taki et al transferred FACS-sorted B–1a cells into SCID mice either alone or with T cells. The T cells enhanced Ig production by B–1a cells and induced switching from IgM to other isotypes, including IgG_1 (115). Similar results were observed in omentum-thymus corecipients in that the addition of the thymic tissue to the graft resulted in substantially increased production of IgG isotypes (76).

Immunoglobulin Rearrangement

Is the machinery that controls Ig heavy and light chain rearrangement different for B–1 and conventional B cells? Both the RAG–1 and RAG–2 gene products are required for successful Ig rearrangement in any type of B cell (118, 119), and no differences have, as yet, been reported for these enzymes in B–1 and conventional B cells. In contrast, terminal deoxynucleotide transferase (TdT), which inserts noncoded nucleotides (N-regions) at the gene segment junctions during rearrangement (120, 121), appears to be absent in the progenitors of the B cells that develop early in fetal life (122, 123). Thus, questions have arisen as to whether all committed progenitors of B–1 cells selectively lack TdT activity and hence whether the absence of Ig N-region insertions is a defining characteristic for this lineage (see Origins, below).

N-REGION INSERTIONS Several groups have demonstrated that fetal and neonatal V_H-D and D-J_H junctions have very few N region insertions whereas most such junctions recovered from adults have longer N regions (124–128). As a consequence of this absence of N-region insertions early in ontogeny, rearrangement of certain V_H-D-J_H gene segments are favored, i.e. those with short sequence homologies (127, 129). Together, these rearrangement mechanisms potentially restrict the early B cell repertoire and thus may have a disproportionate effect on the Ig produced by B–1 cells.

The work published by Rajewsky's group is most informative with respect to N-region insertions because they use PCR amplification to construct cDNA libraries of expressed genes from FACS-sorted B cell subsets (127, 130). Data from these studies show that N-region sequences are rarely inserted at the V_H-D and D-J_H junctions of B–1a cells present in the spleen at four days after birth (average N = 0.6 at the V_H-D and 0.0 at the D-J_H junctions). Peritoneal B–1a cells present at one month of age, in contrast, have more N-region insertions (2.2 at V-D and 0.7 D-J_H) and are intermediate in this sense between the neonatal B–1a cells and conventional B cells isolated from spleen either at one (4.6 V_H-D and 2.8 at D-J_H) or at four months (4.7 at V_H-D and 2.4 at D-J_H). Gu et al also analyzed sequence data from the CH series of B–1 cell lymphomas (97) and showed that many of these neoplasms, which are similar to human B-CLL, lack N-region insertions and hence appear to have arisen early in ontogeny.

For the evaluation of self-replenishing B–1a cells from adults, Gu et al rely on sequence data from hybridomas prepared following LPS stimulation of spleen and PerC of 8-month-old allotype chimeras which, as neonates, were injected with peritoneal cells from 6–10 month old allotype

congenic donors (99). The average length and distribution of the N-region insertions in these hybridomas is similar to N-region size in adult conventional B cells, suggesting that many B–1 cells develop from TdT-expressing B-cell progenitors, which probably begin to function near birth.

The findings reported by Gu et al do not necessarily reflect the size and distribution of N-regions in B–1a cells in normal adult animals, because the highly manipulated B–1 populations in these chimeric mice are likely to be biased. Therefore, we believe it is likely that further analysis will demonstrate that adult B–1 populations, like the FACS-sorted B–1 cells analyzed from 1-month-old animals, have on average more N-region insertions than fetal B–1 populations but fewer such insertions than conventional B cells. Some of the B–1a cells present in the adult may lack N-regions and may have survived via self-replenishment since birth.

Contrasting the representation of N-region insertions in the various B cell lineages in adults may be further complicated by selective processes. A comparison of functional and nonfunctional rearrangements in the 7183 V_H family shows significantly more N-region diversity in rearrangements on the nonfunctional chromosome than on the functional chromosome, in both fetal and adult splenic B cells. Since the analysis of adult splenic B cells most likely is weighted in favor of sequences from conventional B cells, these data suggest (i) that rearrangements associated with a lack of N-region sequences are not restricted to the fetal period; (ii) that rearrangements in both B–1 and conventional B cells may lack N-region sequences; and (iii) that selective forces tend to favor B cells expressing Ig with little or no N-region insertion (131).

J_H PROXIMAL V_H FAMILIES There is considerable evidence demonstrating that the V_H repertoire in fetal and neonatal B and pre-B cell populations is biased towards J_H proximal families while the V_H repertoire in adult splenic B cells is more randomized (normalized) with a heavy expression of genes from the distal (J558) family (132–138). A bias in fetal and neonatal B cells could be related to factors influencing the development of the B–1 cell repertoire, since B–1 cells tend to predominate early in ontogeny and have a functionally restricted repertoire with a high level of self-reactivities. However, mRNA analysis of LPS stimulated conventional and B–1a cells from adults demonstrates that B–1 cells use the whole spectrum of V_H families, without preference for J_H proximal ones (138a,b). The high frequency of V_H11, V_H12 (anti-PtC) and 3609 (anti-thymocytes) gene usage suggests a lack of preference for J_H proximal families by B–1 cells. Short sequence homologies bias junctional recombination of extrachromosomal substrates most readily in cell lines low in TdT expression

(138c). This mechanism may actually be more important in biasing the early Ig repertoire than is chromosome position (129). Homology-directed recombination is likely to be important in generating some B–1 cell specificities such as anti-PC (TI5 idiotype).

Recent evidence indicates that the bias for J proximal V_H families also occurs in developing B cells in adult bone marrow (132, 136). The demonstration that the position-dependent V_H family bias occurs in newly arising B cells at all stages of ontogeny argues strongly that the relatively increased frequency of distal V_H genes in the spleen must reflect the operation of selective (or other) mechanisms that control the entrance or the retention of B cells in the spleen and at other sites.

Selection of Peripheral B–1 and Conventional B Cells

There is no doubt that selection is important in determining the repertoire of both B–1 and conventional B cells. For example, by comparing the V_H repertoire of pre-B and mature B cells from sorted bulk populations, Rajewsky's group has shown that pre-B cells from neonatal liver or adult bone marrow utilize a wide range of V_H genes within the large J558 family whereas the set of V_H genes expressed by peripheral B cells, both B–1a and conventional B, is considerably more restricted (100). Thus, the entry of all B cells into the long-lived peripheral B cell pool either requires positive recruitment or occurs after a negative selection phase.

The selection process begins early in development for both B–1 and conventional B cells, at the stage when pseudo light chain ($\psi L = \lambda 5 + VpreB$) is expressed on immature B cells in conjunction with μ or D_μ proteins (reviewed in 23). The filling of both the B–1 and conventional B cell compartments is impaired in mice made deficient for $\lambda 5$, but not eliminated (139a). The B–1 cell population reaches full size in the $\lambda 5$ knockout mice more slowly than in normal mice, and the conventional B cell population is still reduced five-fold even at 4 months of age. The B–1 cells might simply accumulate better than conventional B cells because of their greater self-replenishing capabilities, although it is possible that B–1 cells are better able to employ alternative differentiation pathways, which are not dependent on $\lambda 5$ protein.

Since B–1 cells develop early in ontogeny, B–1 repertoire differences may at least in part reflect selection by different endogenous antigens and/or immunoglobulins present in the fetus (78, 139). Such selection appears to play a key role in the recruitment of the fetal B cells that produce the germline-encoded antibody specificities prevalent in the B–1 cell population (in neonates and adults). For example, independently rearranged V_H11 and V_H12 genes are expressed in a large series of anti-PtC (an anti-self specificity) hybridomas and lymphomas (98, 109). PCR

amplification of sorted pre-B and B cells from adult bone marrow indicates that functional rearrangements of this V_H11 also occur frequently in conventional B cells; however, these B cells are not found in spleen and thus do not appear to be selected into the peripheral conventional B cell pool (110).

Kearney and coworkers have shown that neonatal treatment with anti-idiotype MAb can deplete specificities and permanently alter the repertoire, as measured by idiotype representation (78). Treatment timing is crucial and related to the normal development of antigen-specific precursors (140). The high interconnectivity (idiotype/anti-idiotype) observed in the early B cell repertoire appears to play a role in these processes, suggesting that production of these kinds of antibodies may be important in establishing the B–1 repertoire expressed in neonatal animals and adults (140–143). Similarly, maternal antibodies transmitted through the placenta and in maternal milk may also influence the characteristics of the B–1 repertoire. These kinds of alterations of the neonatal B–1 repertoire may be particularly important, because they effectively perpetuate neonatal immunologic experience throughout life.

B CELL SELECTION IN TRANSGENIC MICE There are a large number of Ig transgenic mouse strains currently under study in a variety of laboratories (144–151). Many of these strains show B cell developmental defects that alter the relative frequencies of B–1 and conventional B cells and sometimes block rearrangement of endogenous Ig. B–1 cells in several strains have been shown to coexpress endogenous and transgenic Ig or to express mostly endogenous Ig while conventional B cells in the same animals express only the transgenic Ig (56, 152–154). These aberrations in Ig production undoubtedly reflect the selectability of the transgenic Ig and endogenous Ig molecules expressed by individual B cells. Furthermore, they are influenced by the self-replenishing capability of the B–1 cells that are selected into the peripheral pool. However, the operation of these factors does not preclude other differential effects of the transgene, e.g. selective interference with Ig rearrangement in the development of conventional B vs B–1 cells.

THE ORIGINS OF B CELL LINEAGES

Prior to the demonstration that progenitors for B–1 cells are distinct from progenitors for conventional B cells (see Progenitors), there was still room for a "selection-only" hypotheses that viewed B–1 cells as a type of antigen-stimulated conventional B cell, "activated" early in ontogeny and selected to persist via self-replenishment throughout life. This view gained interest

when Wortis and colleagues showed that CD5 expression and other aspects of the B–1a phenotype can be induced by stimulating conventional B cells with anti-IgM antibodies in the presence of certain cytokines (39). However, even this group now agrees that the progenitor studies rule out a simplistic, one-lineage hypothesis (85, 155).

Their current hypothesis (85, 155) proposes two B cell lineages: a fetal lineage, whose TdT-progenitors produce B cells which lack N-region insertions in their rearranged V genes; and an adult lineage, whose TdT^+ progenitors produce B cells which contain N-region insertions in their rearranged V genes. Based on the data from in vitro anti-IgM stimulations, they argue that stimulation of B cells of either lineage with multivalent (cross-linking) TI–2 antigens in the presence of cytokines leads to the expression of CD5 and a shift to the entire B–1a cell phenotype. They then argue that fetal lineage B cells are more likely to be stimulated in this way because the Ig molecules they express, which lack N-region insertions, will be strictly encoded by germline genes evolved to recognize TI–2 antigens such as micro-organismal coat molecules and related self-antigens. Thus, the Wortis group proposes that the B–1a population is largely generated early in ontogeny and persists thereafter by self-replenishment, perhaps stimulated by the self-antigens that initially selected them into the B–1 pool; however, newly differentiated B cells from the adult lineage will enter the B–1 population whenever appropriately stimulated.

We view this "TI–2" model of B cell development as possible but not probable. First, current evidence indicates that B–1 and conventional B cell antibody responses do not segregate with respect to sensitivity to stimulation with TI–2 or any other known classification of antigens (see Responses). Secondly, evidence has yet to be presented demonstrating that the stimulation of conventional B cells that induces expression of the B–1a-like phenotype actually generates functional B–1a cells capable of survival and/or self-replenishment in vivo. In fact, Hayakawa et al have shown that essentially all anti-TNP PFC in the spleen are $CD5^-$ following immunization with the classic TI–2 antigen TNP-Ficoll (105) (see Activation). Third, although more work is required to characterize definitively the frequency of N-region insertion sequences in B–1 vs conventional B cells, current data indicates that a substantially higher representation of N-region sequences in Ig produced by B–1 cells is found in animals over 4 weeks of age (see N-Region) than would be predicted from the observed frequency of new entrants into the B–1 population.

Next, data from the feedback regulation studies demonstrate that the entry of new B cells into the B–1a population in intact adult animals is completely blocked. And finally, although some B–1a cells ($< 10\%$ of the population) appear in bone marrow recipients, their failure to accumulate

over time is inconsistent with the idea that they derive from the adult conventional B cell progenitors, which continuously generate vast numbers of newly rearranged B cells, including some which even have no N-region insertions. Thus current evidence continues to strongly favor the idea that B–1a cells are derived from committed progenitors that do not give rise to conventional B cells; and similarly, that conventional B cells are derived from committed progenitors that do not give rise to B–1a cells.

The Layered Immune System

Current data identify three B cell lineages that appear sequentially, with some overlap, during development. B–1a cells appear sometime after day 16 of fetal life and are readily reconstituted from progenitors in fetal omentum and in fetal and neonatal liver. B–1b cells appear about the same time as B–1a cells (or shortly thereafter). They are readily reconstituted from the fetal and neonatal sources that reconstitute B–1a cells but can also be reconstituted well from progenitors in adult bone marrow. Both B–1a and B–1b cells persist as self-replenishing populations throughout adult life; new entrants into the adult peripheral pool are prevented by a feedback mechanism triggered by the presence of a mature B–1 population. Conventional B cells, in contrast, begin to appear during the post-natal period, are readily replenished in situ from undifferentiated progenitors, and are reconstituted in transfer studies from progenitors present in both fetal and adult sources.

The recognition of distinct B-cell lineages could be strictly interpreted within the framework of B cell development; however, the progenitor studies with FACS-isolated HSC populations from fetal and adult sources suggest a broader context for consideration of these findings (Figure 3). These populations contain pluripotent stem cells that, by definition, also give rise to T cells, erythrocytes, and myeloid cells. Thus the demonstration that HSC from fetal and adult sources give rise to distinct B cell lineages suggests the existence of similar lineages of other kinds of differentiated hematopoietic cells. Since certain of these lineages have already been identified (7, 18), these considerations lead us to propose that evolution has created a layered immune system by successively adding developmental lineages that provide progressively more complex functions (44, 156).

The parallel developmental patterns and repertoires exhibited by T- and B-cell populations/lineages suggest that B–1a cells and early $\gamma\delta$ (Vγ3) T cells represent the most primitive "layer" of this immune system. Subsequent layers then might link B–1b cells with of Vγ4 cells (157, 158) and, finally, conventional B cells with the remainder of the T cell populations. Data supporting this concept have been reviewed elsewhere (62, 156, 158). For example, $\alpha\beta$ T cells, like conventional B cells, appear around birth

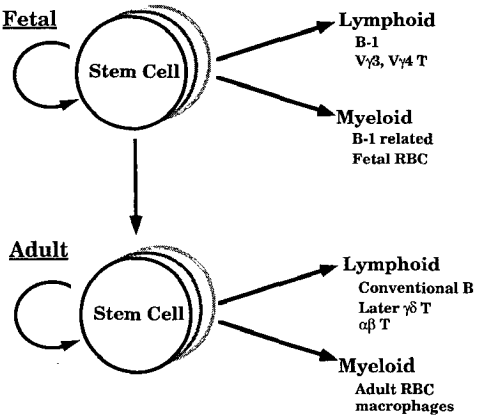

Figure 3 The layered immune system. Transfer studies demonstrate that the stem cells present in fetal life give rise to different sets of hematolymphoid cells than those stem cells present in the adult. The distinct progenitors have been demonstrated for murine B cells (14, 72, 73) and T cells (7, 18), and sheep erythrocytes (10–13).

and become predominant as the animal matures. Both $\alpha\beta$ T cells and conventional B cells, which circulate throughout the animal and predominate in secondary lymphoid organs, can be replenished throughout life by de novo differentiation from stem cells in the bone marrow.

Functional considerations suggest that B–1 cells and early $\gamma\delta$ T cells, by nature of their repertoire and anatomical location, may create a first line of defense against invading pathogens. B–1 cells produce a more restricted set of low-affinity, broad-specificity germline antibodies that react with ubiquitous microorganisms, whereas conventional B cells produce a large, more diverse set of antibodies capable of specific high affinity interactions with particular pathogens. Similarly, the repertoire of the early $\gamma\delta$ T cells is considerably more restricted than the diverse repertoire of $\alpha\beta$ T cells. Thus, the functional distinctions among layers in the immune system are visible both phylogenetically and ontogenically.

In sum, the evolution of the immune system appears to have brought into existence a series of stem cells that sequentially give rise to lymphocytes that are similar to their predecessors but may have added (or lost) functional capabilities. Because the evolutionary success of the latest layer depends on its ability to introduce a selective advantage, each new layer can be expected to increase the sophistication of the immune system with respect to its ability to efficiently protect the animal against challenges within its environment. This concept of an evolutionarily layered immune system presents a framework that unifies data from T and B lineage studies and offers a model that can guide future work.

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TRANSCRIPTIONAL REGULATION OF T CELL RECEPTOR GENES

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KEY WORDS: transcription, T lymphocyte, gene expression, ets, GATA, TCF–1, LEF–1, CREB/ATF

Abstract

The diverse lineages of the mammalian hematopoietic system including both B and T lymphocytes are derived from a single mesodermal progenitor, the pluripotent bone marrow stem cell. The coordinate transcriptional regulation of sets of lineage-specific genes is one of the important molecular mechanisms underlying hematopoietic lineage determination and differentiation. The immunoglobulin and T cell receptor (TCR) genes have been used as model systems to study lineage-specific transcriptional regulation during lymphoid development. This review summarizes our current understanding of the regulation of TCR gene expression during thymocyte ontogeny. Expression of each of the TCR genes is controlled by T cell–specific transcriptional enhancers that bind partially overlapping sets of ubiquitous and lymphoid-specific transcription factors. These include members of both the ATF/CREB family of basic-leucine zipper proteins and the Ets protooncogene family, as well as the T cell–specific zinc finger transcription factor, GATA–3, and the T cell–specific high mobility group proteins TCF–1 and TCF–1α/LEF–1. The identification of binding sites for these same transcription factors in a number of additional T cell–specific genes suggests that they may play important roles in the coordinate regulation of gene expression that specifies the development of the T cell lineages. Recent studies of the TCR α and γ genes have suggested that negative regulatory elements or transcriptional silencers may also play an

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important role in controlling the lineage-specific expression of these genes. On going studies are designed to clarify the role of each of the TCR enhancer binding proteins in regulating T cell development in vivo, to more precisely define the interactions between the TCR enhancer binding proteins, and to elucidate the molecular mechanisms underlying transcriptional silencer activity.

INTRODUCTION

The vertebrate hematopoietic system is composed of multiple specialized cell lineages that subserve diverse functions including oxygenation, coagulation, and inflammation. Thus, it is remarkable that all of the mature hematopoetic effector cells are derived from a single progenitor, the pluripotent bone marrow stem cell. The diverse functional capacities of the hematopoietic lineages are determined by their expression of distinct sets of tissue-specific genes. These include specialized cell-surface receptors as well as lineage-specific enzymes and structural proteins. The tissue-specific expression of many of these genes is controlled at the level of transcription. Therefore, an understanding of hematopoietic cell function and development must, at some level, be based upon elucidating the molecular mechanisms that control tissue-specific transcription during hematopoiesis. In addition to increasing our understanding of hematopoietic cell function, such studies might also establish novel paradigms concerning the mechanisms involved in positively and negatively regulating gene expression during mammalian development and, additionally, might help to clarify the molecular defects underlying certain inherited disorders of hematopoietic function.

One of the important features of the mammalian hematopoietic system is its capacity specifically to recognize and respond to foreign antigens present on invading pathogens and tumor cells. This function is subserved in large part by the lymphoid system which is composed of two major cell lineages—B cells and T cells. Cells of the two lymphoid lineages express distinct but related cell-surface antigen receptors and thereby recognize different types of antigens. B cells express cell-surface immunoglobulin and recognize soluble antigens, whereas T cells express cell-surface T cell receptor (TCR) molecules and recognize antigenic peptides bound to major histocompatibility molecules on the surface of antigen presenting cells (1–5). At least two lineages of T cells can be distinguished on the basis of their differential expression of T cell receptors (2, 4–6). The major subset of circulating peripheral blood T cells which includes all cells of the classical helper and cytotoxic T phenotypes expresses the heterodimeric α/β TCR (1, 4, 5). In contrast, a minor population of circulating T cells express the

distinct heterodimeric γ/δ TCR (2, 7–9). The function of these γ/δ T cells remains unclear. However, their localization at epithelial surfaces suggests that they may play a role in responding to the invasion of pathogens at these surfaces (6, 9). Several models have been proposed to explain the relationship between the α/β and γ/δ T cell lineages. Initial studies suggested that α/β T cells might be derived from cells that had previously undergone nonproductive rearrangements of their TCR γ and δ genes (10, 11). However, recent work is much more consistent with a model in which α/β and γ/δ T cells represent distinct lineages that separate prior to the rearrangement of their TCR genes (12). This latter model if correct suggests the existence of lineage-specific mechanisms that control the rearrangement and expression of the TCR genes. An understanding of these mechanisms is therefore central to understanding the differentiation and separation of the two T cell lineages.

The immunoglobulin (Ig) and TCR genes are structurally related; each is composed of multiple variable, diversity (in some cases), and joining gene segments that undergo rearrangement during lymphocyte ontogeny (13, 14). Despite the finding that a common recombinase is responsible for both Ig and TCR gene rearrangements (15), the rearrangement and expression of these genes is tightly regulated and lineage-specific with TCR gene expression limited to T cells and Ig rearrangement limited to B cells (16). Similarly, the rearrangement and expression of the TCR α and δ genes are lineage-specific, with TCR δ gene expression limited to γ/δ T cells and TCR α gene rearrangement and expression limited to α/β T cells (17). Experiments by Alt and coworkers have suggested that the rearrangement of the Ig and TCR genes may be regulated by their lineage-specific transcriptional activation (18). Thus, an understanding of the mechanisms that control the lineage-specific transcription of these genes may also help to explain their tissue-specific patterns of rearrangement.

Given their important functions and tissue-specific patterns of expression, the TCR and Ig genes have been studied extensively as models of lineage-specific transcriptional regulation during mammalian hematopoiesis. As described below, these studies have resulted in the identification of novel families of lymphoid-specific transcription factors, some of which appear to play an important role in regulating B and T cell development. In addition, they have elucidated several new principles governing the interactions between enhancer binding proteins, and the way that these interactions may regulate the basal transcription machinery. Finally, these studies have increased our understanding of the molecular mechanisms that regulate the expression of lymphotropic viruses including the human immunodeficiency viruses.

This review summarizes our current understanding of the molecular

mechanisms regulating the expression of the human and murine TCR genes, with particular emphasis on four central issues: (i) the molecular mechanisms regulating the lineage-specificity of expression of the TCR genes, (ii) the relative importance of positive and negative *cis*-acting transcriptional regulatory elements in controlling TCR gene expression, (iii) the molecular mechanisms underlying changes in TCR gene expression during both thymocyte development and the activation of peripheral T cells, and (iv) the identification of transcription factors that regulate the expression of multiple T cell genes (including the TCR genes) during thymocyte ontogeny. Given the large number of contributors to this field it is impossible to include all citations. I apologize for any unintentional omissions. For further information relevant to this topic, the reader is referred to several recently published reviews of T cell development (3, 10, 19, 20), TCR gene structure and function (1, 2, 4), and TCR and Ig gene regulation (16, 21).

STRUCTURE AND PATTERNS OF EXPRESSION OF THE TCR GENES

The TCR α, β, and γ genes are located at distinct chromosomal loci (14q11, 7p15, and 7q32, respectively, in the human), and each is composed of multiple gene segments that rearrange during thymocyte ontogeny (1, 2, 4, 13, 22–26) (Figure 1). Interestingly, the TCR δ gene lies between the Vα and Jα gene segments within the TCR α locus (27) (Figure 1) such that productive rearrangements of the TCR α gene must be accompanied by deletion of the entire TCR δ locus. The four TCR genes display distinct and highly regulated patterns of rearrangement and expression during thymocyte differentiation (28–34). The TCR δ gene is rearranged and expressed beginning on day 14 in the mouse fetal thymus. This is followed on days 15 and 16 by TCR β and γ rearrangement and expression. Finally the TCR α gene undergoes rearrangement and expression beginning on day 17 of fetal life and coinciding with cell-surface expression of the CD3/TCR α/β complex on thymocytes. Thus, TCR α gene rearrangement and expression appear to be the rate-limiting steps in the expression of the CD3/TCR complex at the cell-surface.

In addition to the differences in their temporal patterns of expression, the TCR genes are differentially rearranged and expressed in the α/β and γ/δ T cell lineages. For example TCR α gene rearrangement and expression is restricted to α/β T cells (17), most of which display deletions of the TCR δ locus on both chromosomes (35). This finding has led to the hypothesis that determination of the α/β T cell lineage involves a mechanism that results in the deletion of both TCR δ loci prior to TCR α gene rearrange-

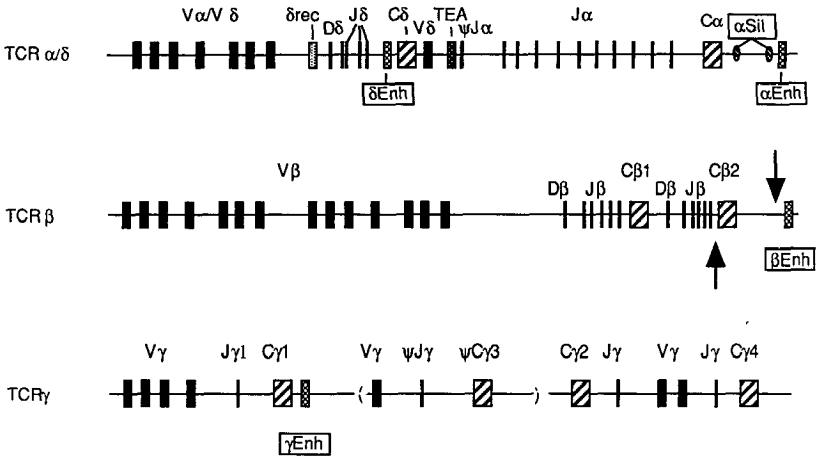

Figure 1 TCR transcriptional regulatory sequences. Schematic illustrations of the human TCR α/δ, and β loci and the murine TCR γ locus. The known TCR enhancers and silencers are labelled. Drawings are not to scale. The precise number of TCR α, β, and γ V region gene segments is unknown. For convenience the separate exons of the variable and constant region gene segments are not shown. The orientation of the murine $\Psi C\gamma 3$ gene segment relative to that of $C\gamma 1$ and $C\gamma 4$ is unknown. DNaseI hypersensitive sites 5′ of the TCR β enhancer are shown by arrows.

ment (35, 36). Similarly, TCR δ gene rearrangement and expression are limited to γ/δ T cells (37). The patterns of rearrangement and expression of the TCR β and γ genes are more complex. TCR β gene rearrangements are seen in both α/β and γ/δ T cells (38). However, full length TCR β transcripts are rarely observed in γ/δ T cells, probably reflecting the fact that most TCR β gene rearrangements in γ/δ cells are D-J rearrangements (without concomitant V-DJ rearrangement). There is little evidence for negative regulation of TCR β gene transcription in γ/δ T cells. In fact, recent studies of TCR β transgenic mice have shown that both γ/δ and α/β T cells express the TCR β transgene (39, 40). In contrast to the patterns of rearrangement and expression of the α,β, and δ TCR genes, the TCR γ gene undergoes V-D-J rearrangement in many α/β T cells (41–43). In as many as one third of cases this rearrangement is productive and could result in a functional TCR γ transcript (44, 45). However, despite these frequent rearrangements, TCR γ mRNA is not detectable in freshly isolated α/β T cells (41). This finding provided the first evidence for negative transcriptional regulation of the TCR γ locus in non-γ/δ T cells, a model discussed in detail below. In summary, the four TCR genes display distinct temporally regulated and lineage-specific patterns of expression. These patterns appear to be controlled at the levels both of rearrangement and

transcription. However, as described above, it is likely that patterns of rearrangement are determined at least in part by the regulated transcriptional activation of the unrearranged TCR genes. Thus, an understanding of the mechanisms regulating lineage-specific transcription may be important in elucidating the mechanisms controlling the lineage-specific patterns of rearrangement of the TCR genes during thymocyte differentiation.

EVIDENCE FOR POSITIVE AND NEGATIVE REGULATION OF TCR GENE EXPRESSION

Classical models of tissue-specific transcriptional regulation involve the lineage-specific expression or function of transcriptional activating proteins. However, evidence from a number of systems suggests that negative regulation also plays a role in determining tissue-specific expression of TCR genes (46). MacLeod et al (46) reported that hybrids formed between TCR β^- and TCR β^+ variants of murine T lymphoma cell lines often extinguish TCR β mRNA expression, and that this negative regulatory effect can be partially reversed by treatment of the hybrids with cycloheximide. Similarly Meda et al (47) reported that fusion of a TCR β^+, TCR α^-, TCRγ^+ murine thymoma with a TCR β^-, TCRα^+, TCRγ^- thymoma resulted in hybrids that suppressed expression of both the TCR α and γ genes, but continued to express TCR β mRNA. Treatment of the hybrids with PMA caused reexpression of the TCR α gene, but failed to derepress TCR γ gene expression. These experiments demonstrate that, at least in murine lymphoma cell lines, TCR α, β, and γ gene expression can be negatively regulated by multiple distinct mechanisms, although the level(s) and mechanism(s) responsible for this negative regulation remain unclear. Cell fusion experiments also provide evidence for positive regulation of TCR gene expression. For example, Marolleau et al (48) found that cell hybrids formed between two TCR γ^- immortalized murine cell lines activate TCR γ transcription, suggesting *trans*-complementation by positively acting transcription factors.

Transgenic experiments have shed further light on the question of negative regulation of TCR gene expression. Tonegawa and coworkers (33, 49) examined T cell development and TCR gene expression in TCR γ/δ double transgenic mice made with different TCR γ transgenes containing distinct amounts of 5′ and 3′ flanking sequences. TCR γ/δ double transgenic mice produced with a 40-kb Vγ4Jγ1Cγ1 transgene contained normal numbers of α/β T cells that had extinguished expression of the TCR γ transgene. In contrast γ/δ double transgenic mice produced with a 15-kb version of the same transgene that lacked both 5′ and 3′ flanking sequences contained

severely reduced numbers of α/β T cells. Moreover, α/β T cells from single transgenic mice produced with the 15-kb TCR γ transgene expressed high levels of TCR γ transgene mRNA. Taken together these data are consistent with a model in which the flanking sequences of the TCR γ transgene contain *cis*-acting sequences responsible for negatively regulating TCR γ gene expression in α/β T cells in vivo.

CIS-ACTING SEQUENCES THAT REGULATE TCR GENE EXPRESSION

TCR β Gene Expression

Expression of the TCR β gene is controlled by the interaction of promoters located in the 5′ flanking region of the Vβ genes (Vβ promoters) with a potent transcriptional enhancer located 3.5–5.0 kb 3′ of the Cβ2 gene segment (50–56) (Figure 1). The location of this 3′ enhancer which has been conserved between mouse and human ensures that it is not deleted by the TCR β gene rearrangements. Transient transfection assays have revealed that the Vβ promoters themselves are essentially inactive in both T cells and non-T cells (50, 52, 55–57). Thus, these promoters require the activity of the 3′ enhancer for significant transcriptional activity. An important question concerns the cell-lineage specificity of activity of both the Vβ promoters and the 3′ enhancer. Although early studies suggested that the Vβ promoters themselves might confer tissue specificity on TCR β gene expression (51), more recent work has convincingly demonstrated that these promoters can be activated in a variety of T cells, B cells, and nonlymphoid cells by heterologous enhancers (50). Indeed, a human Vβ promoter alone has been reported to display higher transcriptional activity in B cells than in T cells although its activity alone was low in both cell lines (50). In contrast to the Vβ promoters, the 3′ TCR β enhancer is relatively T cell–specific both in combination with its own and with heterologous promoters (50, 52, 54, 55). Recent studies have suggested that the enhancer may consist of a core element which is fully active in T cells and partially active in some B cells, along with a flanking element that restricts the activity of the full enhancer to T cells (i.e. a transcriptional silencer element) (53, 54). This silencer has not yet been precisely localized or characterized. Finally, although the 3′ enhancer is itself T cell specific and is required for high level transcription from a Vβ promoter, some evidence suggests that the combination of the 3′ enhancer and a Vβ promoter is more active in T cells than is the 3′ enhancer in combination with heterologous promoters (50). Thus, the promoter, although not itself T cell specific, may potentiate the activity of the 3′ enhancer in T cells. Finally, it should be emphasized that the TCR promoter/enhancer combination, although T-

cell specific, is equally active in α/β and γ/δ T cells in transient transfection assays (50). This is consistent with transgenic experiments in which the expression of a fully rearranged TCR β transgene containing the TCR β promoter and enhancer was observed in all T cells (39, 40). Thus, it remains unclear why V-D-J rearrangements of the TCR β gene are normally restricted to α/β T cells (this point is discussed further below).

The murine and human TCR β promoters and enhancers have been subjected to detailed deletional analysis and assayed for their ability to bind nuclear protein complexes from a variety of lymphoid and non-lymphoid cell lines (50, 54, 56, 57). Deletional analyses of murine TCR β promoters demonstrated that essentially full promoter activity was retained by a fragment containing only 84 bp of 5′ flanking sequence (56, 57). DNA sequence analysis of multiple human and murine Vβ promoters revealed a conserved decamer motif usually located within 100 bp of the transcriptional start site (56, 57). Subsequent studies demonstrated that this decamer corresponds to a binding site for the CREB/ATF family of leucine zipper transcription factors, a binding site also found in both the TCR β and α enhancers (see below) (50). Mutational analysis of a single murine Vβ promoter suggests that the decamer motif is required for promoter activity (56); however, similar studies on several human Vβ promoters suggest that in some cases this motif is not essential for transcriptional activity at least when assayed in combination with the 3′ TCR β enhancer (I.C. Ho, J. Leiden, unpublished data). Additional nuclear protein binding sites have been detected in human Vβ promoters (58). However, the functional significance of these sites remains unclear as they have not been systematically mutated.

Deletional analyses of the human and murine 3′ TCR β enhancers suggest that the core enhancer is contained on a 400-bp fragment composed of at least 3–5 nuclear protein binding sites (50, 54) (Figure 2A). Detailed mutational analyses of the human 3′ flanking enhancer demonstrated that three of these sites (called Tβ2, Tβ3, and Tβ4) are required for full enhancer function in mature T cell tumor cells (50). Interestingly, each of these binding motifs has been highly conserved in the mouse and human enhancers. Several additional binding sites (Tβ1 and Tβ5) do not appear to be required for enhancer activity in mature T cells. However, it remains possible that these elements regulate enhancer function at earlier stages of T cell development. The identities of several of the TCR β enhancer binding proteins have recently been established. These binding activities are summarized schematically in Figure 2A and discussed in more detail below. They include a GATA–3 binding site (in Tβ2), a CREB/ATF binding site (in Tβ2), an Ets–1 binding site (in Tβ3) and an LEF–1/TCF–1 binding site (in Tβ5) (21, 50). In addition the enhancer has been shown to bind to

transcription factors, μEBP-E and κE2, both of which are known also to bind to Ig enhancers (52, 54).

Given the relatively T cell–specific activity of the TCR β enhancer, it is noteworthy that none of the nuclear protein complexes previously shown to bind to the TCR β enhancer are T cell specific (50, 54). There are several possible explanations for this apparent paradox. It remains possible that important T cell–specific binding activities have not been detected by the DNaseI footprint and EMSA analyses employed in previous studies. Alternatively, T cell–specific factors that interact with ubiquitous DNA binding proteins but do not themselves bind to DNA may determine the specificity of enhancer function. It is also possible that T cell–specific post-translational modifications of ubiquitous transcription factors restrict their activity in vivo. Moreover, as discussed in more detail below, there may be additional positive and/or negative transcriptional regulatory sequences that are important in restricting the activity of the enhancer. Thus, for example, there may be positive regulatory elements analagous to the locus control regions in the globin gene cluster that play an important role in determining lineage-specific expression. Conversely, there may be silencers that limit the activity of the enhancer to T cells. Finally, it is possible that the enhancer is active in multiple lymphoid lineages but that TCR β gene expression is limited to α/β T cells because it is only in that lineage that Vβ promoters are brought into proximity with the enhancer, i.e. that lineage-specific rearrangement rather than transcriptional enhancer activity per se determines the lineage-specific expression of the TCR β genes. This last model would be consistent with previous findings that showed that rearranged TCR β transgenes are transcriptionally active in all T cells and also display variable activity in B cells in vivo (39, 40). These explanations are not mutually exclusive, and multiple mechanisms may underlie the observed lineage-specificity of TCR β gene rearrangement and expression.

The possibility of additional *cis*-acting regulatory sequences in the TCR β locus (Figure 2A) has been raised by recent reports of additional DNaseI hypersensitive sites within the locus, and by the finding of additional nuclear protein binding sites that correspond to these hypersensitive sites (59–61). Thus, for example, Hashimoto and coworkers (59) have described a DNaseI hypersensitive site located approximately 400 bp 5′ of the TCR β enhancer in the mouse. This site which they termed DHD2 was particularly interesting because it was only present in T cells that expressed TCR β mRNA. In addition, sequences surrounding this site contained potential binding sites for several previously described transcription factors. However, the functional significance of the site remains unclear because it lacks transcriptional enhancer activity by itself(59). Nevertheless, it

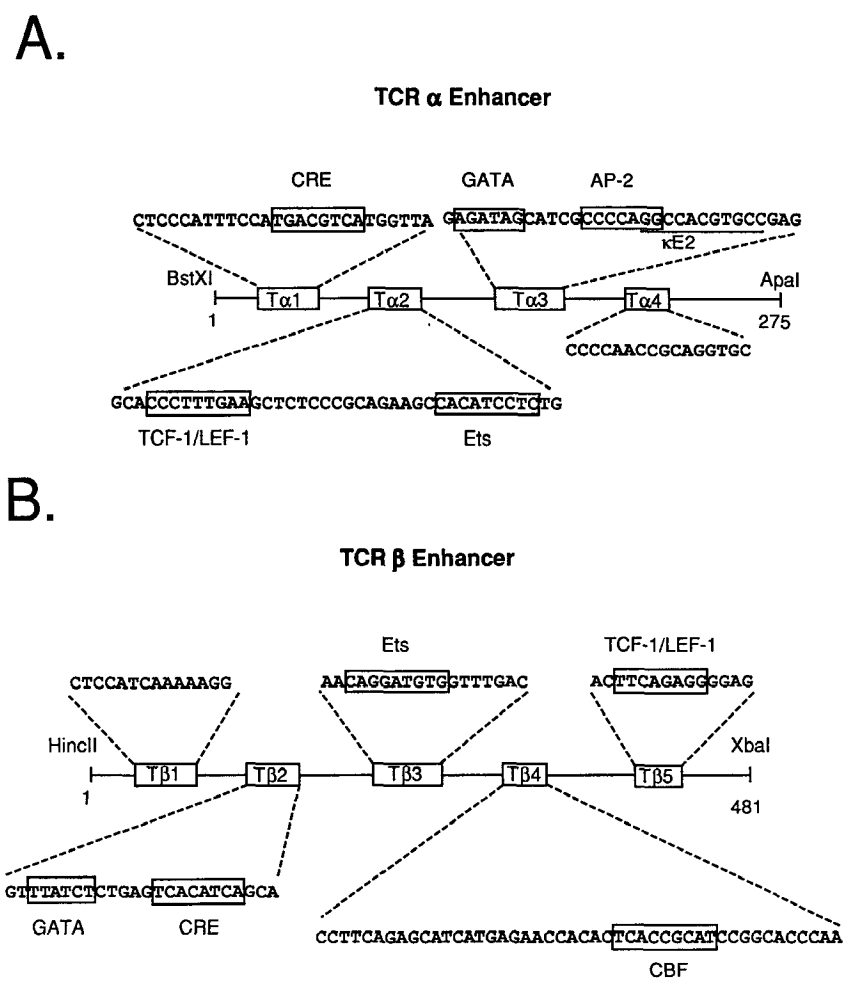

Figure 2 Structures of the TCR enhancers. The human TCR α, β, and δ and murine TCR γ enhancers are shown schematically along with the names and DNA sequences of their previously identified nuclear protein binding sites. Known transcription factor binding sites are boxed and labelled (see text for a description of these sites and their cognate transcription factors). The sizes of the enhancers in bp are shown below each map.

remains possible that it modulates the activity of the TCR β promoters or enhancers in vivo. A second pair of DNaseI hypersensitive sites located in the Jβ2-Cβ2 intron has recently been described by two groups (60, 61) (Figure 2A). These sites were T cell–specific and were shown to bind nuclear protein complexes from T cells. Their functional significance,

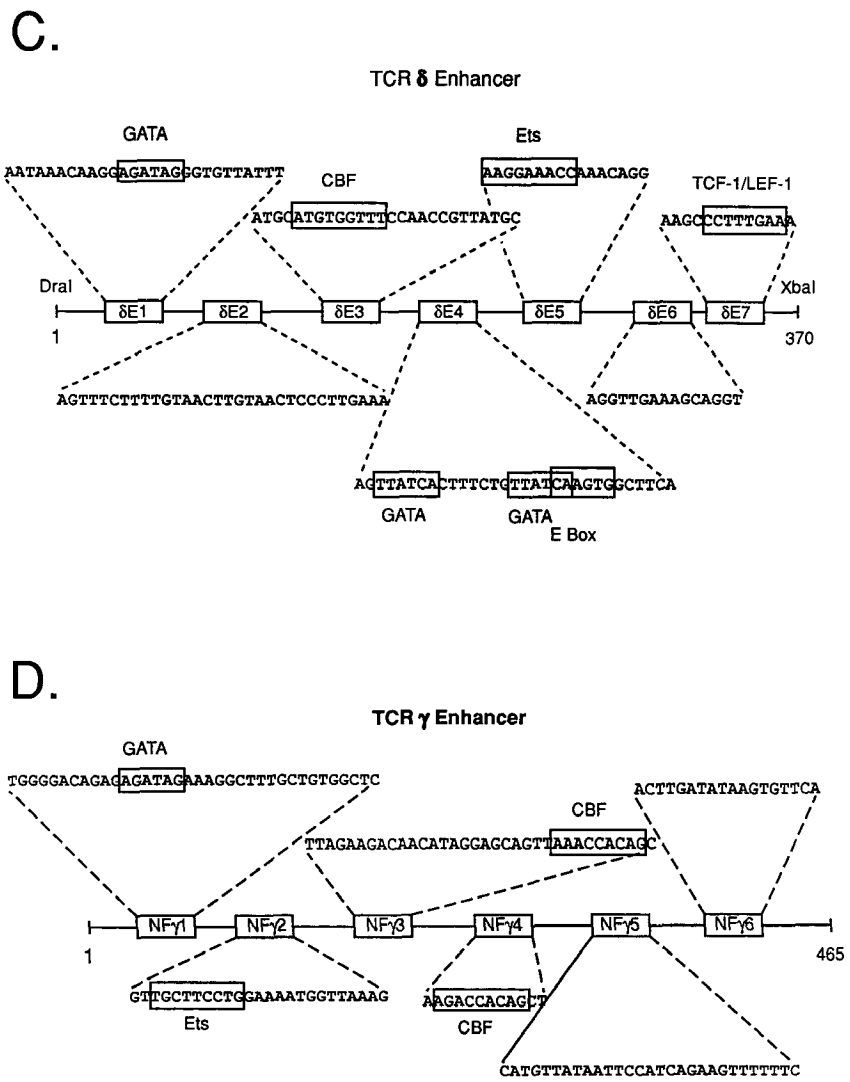

Figure 1 (*continued*)

however, remains unknown as they do not themselves display transcriptional enhancer activity. Finally, Diamond et al (51) described a T cell–specific transcriptional enhancer located 570 bp 5′ of the transcriptional start site of the human Vβ8.1 gene. The significance of this enhancer is unclear because it had little activity (less than 3–5-fold increases in transcription when paired with its own core promoter), and because

similar activities have not thus far been detected in several other Vβ promoters (50, 56). It would be interesting to study the role of each of these additional sequences by making TCR β transgenic animals using transgenes containing various combinations of these sequences together with Vβ promoter and enhancer sequences.

TCR α Gene Expression

There are several striking similarities between the molecular mechanisms that regulate TCR α and β gene expression in α/β T cells. Like the TCR Vβ promoters, TCR Vα promoters display little intrinsic transcriptional activity and are not tissue-specific when linked to a variety of ubiquitously active enhancers (62, 63). As is the case for TCR β gene expression, high level tissue-specific TCR α gene expression is regulated by a potent T cell–specific transcriptional enhancer located 3–4.5 kb 3′ of the single Cα gene segment in both the murine and human TCR α loci (62, 64) (Figure 1). The minimal human TCR α enhancer has been localized to a 275-bp fragment whose nucleotide sequence is more than 90% conserved in mouse and human (62). In transient transfection assays this enhancer was required for high-level transcription from a Vα promoter and also stimulates high-level T cell–specific transcription from heterologous promoters such as the minimal SV40 or *c-fos* promoters (62–64).

The human TCR α enhancer contains four functionally significant nuclear protein–binding sites, called Tα1-Tα4 which are now known to bind at least seven T cell nuclear protein complexes (62, 65) (Figure 2A). A similar analysis of the murine enhancer identified nuclear protein binding sites corresponding to Tα1 (NFα2) and Tα2 (NFα3 and NFα4) (64). The Tα3 and Tα4 sites were not detected in the analysis of the murine enhancer because the fragment used for DNase1 footprinting did not extend 3′ of Tα2. Mutational and deletional analyses of the human TCRα enhancer demonstrated that, at least in the Jurkat T cell line, the minimal TCR enhancer is contained within a 116 bp fragment that includes only the Tα1 and Tα2 nuclear protein-binding sites (63). By contrast, a fragment containing the Tα3 and Tα4 elements alone displayed low-level enhancer activity (6–10-fold as compared with 50–100-fold for the Tα1 + Tα2 containing fragment) (63). Both Tα1 and Tα2 were required for the activity of the minimal TCR α enhancer. In addition, the spacing between Tα1 and Tα2 was critical for enhancer function; reducing the normal 20 bp spacing to 15 bp eliminated enhancer activity, as did increasing the spacing to more than 85 bp. In contrast, the phase of the Tα1 and Tα2 elements on the DNA helix did not appear to be critical. Finally, the Tα3 and Tα4 elements, while themselves not possessing high-level enhancer activity, could compensate for mutations in Tα1 or Tα2 that abolished enhancer

activity (63). Thus, Tα3 and Tα4 represent redundant elements, at least as assayed in Jurkat T cells. It remains possible however, that Tα3 and/or Tα4 modulate TCR α enhancer function at some earlier stage in T cell development, or in response to specific extracellular signals. These functional analyses suggest that the TCR α enhancer is composed of multiple, partially redundant, nuclear protein–binding sites. Interactions between the proteins that bind to these different sites appear to be important for enhancer function.

DNA sequence analysis of the nuclear protein binding sites of the murine and human TCRα enhancers revealed several previously described transcriptional regulatory motifs (50, 62–64) (Figure 2A). For example, Tα1 contains an evolutionarily conserved cAMP response element (TGACGTCA). Similarly, Tα3 contains overlapping GATA (WGATAR), AP–2 (CCCCAGGC) and κE2 (E Box) (AGGCCACGTGCCGA)-like motifs. Finally Tα2 contains potential TCF–1/LEF–1 and Ets binding sites. In contrast, Tα4 does not contain previously identified nuclear protein-binding sites. Electrophoretic mobility shift assays (EMSAs) using synthetic oligonucleotides corresponding to Tα1-Tα4 demonstrated that Tα1 binds a set of four to six ubiquitously expressed cAMP response element binding (CREB) proteins (50). Binding of each of these proteins was inhibited by mutations within the core of the CRE, and no Tα1-binding proteins were detected that recognized non-CRE sequences (I-C. Ho, J. M. Leiden, unpublished). Similar EMSAs demonstrated that Tα2 binds at least four nuclear protein complexes (63). One of these is T cell–specific, while a second is present in all T cell, and some B cell, nuclear extracts. Tα3 binds three nuclear protein complexes, one of which is T cell–specific. Finally, Tα4 binds three ubiquitously expressed nuclear protein complexes. Interestingly, none of these experiments identified α/β T cell lineage–specific nuclear protein complexes (63).

The apparent lack of α/β T cell–specific TCR α enhancer binding proteins raised important questions concerning the molecular mechanisms that restrict expression of the TCR α gene to α/β T cells. Winoto & Baltimore (66) subsequently reported that the core murine TCR α enhancer is equally active in α/β and γ/δ T cells. They also identified several lineage-specific transcriptional silencers located 5′ of the murine TCR α enhancer (Figure 2A) which are required to restrict the activity of the enhancer to the α/β T cell lineage. In transient transfections these silencers functioned in an orientation- and position-independent manner to extinguish the activity of the TCR α enhancer in all non-α/β cells (including γ/δ T cells). Moreover the silencers restricted the activity of normally ubiquitously active enhancers such as the Moloney enhancer to non-α/β T cells. As the authors point out, (66) the mechanism by which the TCR α silencers extinguish TCR α

transcription remains unclear. Although both silencer elements bound nuclear proteins, mutagenesis of their nuclear protein binding sites did not alter silencer function. Moreover, it is unlikely that the silencers bind repressor proteins that are present in all non-α/β T cells, because the TCR α gene can be transcribed in hybrid cell lines formed between α/β and γ/δ T cells (33). Finally, although negative regulatory elements also exist 5′ to the human TCR α enhancer, these elements are not in themselves sufficient to restrict the activity of the human TCR α enhancer to α/β T cells, and they fail to extinguish the activity of ubiquitously active enhancers such as the Rous sarcoma virus LTR to α/β T cells (I.-C. Ho, J. Leiden, unpublished data). A better understanding of the role and mechanisms by which transcriptional silencers regulate TCR α gene expression awaits more detailed deletional, mutational, and nuclear protein binding analyses, as well as transgenic studies using transgenes containing or lacking different putative silencers.

TCR γ Gene Expression

Two groups have independently identified a transcriptional enhancer located 3 kb 3′ to the Cγ1 gene segment in the mouse (67, 68) (Figure 1). Deletional analyses yielded equivocal results about the precise location of the enhancer with different minimal enhancers detected in transient transfections of different cell lines. For example a 400-bp EcoRV/PvuII fragment showed maximal enhancer activity when assayed in Peer γ/δ T cells or Jurkat α/β T cells, but was relatively inactive when assayed in EL4 α/β T cells. In contrast, a larger (approximately 1 kb) NcoI/HindIII fragment that contains the EcoRV/PvuII fragment was active in EL4 T cells. These differences may reflect the presence of multiple partially redundant nuclear protein binding sites in the TCR γ enhancer in conjunction with the expression of limited and distinct subsets of enhancer binding proteins in different T cell lines (68). The TCR γ enhancer displayed T cell–specific activity but was not active as a monomer in all T cell lines. This finding suggests the existence of additional *cis*-acting regulatory sequences that are required for TCR γ gene expression in some T cell subsets. Alternatively it may reflect an artifact of the transient transfection assays used to analyze enhancer activity. Interestingly, like the TCR β enhancer, the TCR γ enhancer was equally active in α/β and γ/δ T cells (68). Transgenic experiments strongly support the importance of this enhancer for TCR Cγ1 gene expression, because transgenes containing the enhancer are expressed at high levels in both α/β and γ/δ T cells, whereas transgenes that are lacking the enhancer are not expressed in the T cell lineage (68).

DNase I footprint analyses of the TCR γ enhancer revealed six nuclear

protein binding sites which were equivalently protected by nuclear extracts from both α/β and γ/δ T cells (68) (Figure 2B). Whether any of these sites bind lymphoid or T cell–specific nuclear proteins remains unknown. One of the nuclear protein binding sites (NFγ2) contained a consensus Ets binding motif, while two others (NFγ3 and NFγ4) were highly related to the Tα2B sequence from the TCR α enhancer and to the core binding factor (CBF) site from the Moloney virus LTR. DNA sequence analysis revealed additional motifs that were highly related to κE2 binding sites of the Igκ enhancer, an AP–2 site, a topoisomerase II site and multiple potential matrix attachment regions (67, 68). The functional significance of each of these sites awaits detailed deletion and mutagenesis studies. The finding that the minimal TCR γ enhancer was equally active in α/β and γ/δ T cells, along with the fact that the rearranged TCR γ1 genes are not normally expressed in freshly isolated α/β T cells suggested the existence of transcriptional silencers that restrict TCR γ1 gene expression to γ/δ T cells. As described above, Tonegawa and coworkers (49) have used transgenic experiments to provide direct evidence for such a TCR γ silencer, which is probably located 3′ of the TCR Cγ1 gene segment. The precise localization and mechanism of action of this silencer(s) requires further study. In addition, it will be important to determine whether the Cγ2 and Cγ4 gene segments each contain distinct 3′ enhancers and/or silencers. Finally, because the different TCR γ gene segments undergo a precisely orchestrated pattern of rearrangement during thymic ontogeny (20, 28, 41), we need to better understand the role of specific Vγ promoters in controlling the temporal pattern of TCR γ rearrangement and expression during thymocyte development.

TCR δ Gene Expression

Because the TCR δ gene is embedded within the TCR α locus (27) (Figure 1), it was initially unclear whether TCR δ gene expression was regulated by the TCR α enhancer or was controlled by an independent transcriptional enhancer located within the TCR δ locus itself. This question was resolved by Redondo et al (69) who identified and characterized a potent transcriptional enhancer located within the Jδ3-Cδ intron of the human TCR δ gene (Figure 1). Subsequent studies independently confirmed the identity of this enhancer in both mouse and human (70, 71). Thus, the TCR δ gene is apparently unique among the TCR genes in lacking a 3′ enhancer. Transient transfections demonstrated that the TCR δ enhancer is T cell–specific but is active with a variety of promoters in both TCR α/β and γ/δ cell lines (69). So far, there is no evidence of lineage-specific transcription of the TCR δ gene.

DNaseI footprint analyses of the human TCR α enhancer revealed seven

nuclear protein binding sites (called δE1-δE7) (69) (Figure 2B). It remains unclear which of these, if any, bind T cell- or lymphoid-specific nuclear proteins. Preliminary deletional analyses suggested that the core enhancer is composed of sequences that include the δE3-δE6 nuclear protein binding sites. However, a detailed mutagenesis analysis of this enhancer has not yet been reported. Sequence comparisons revealed several interesting motifs within the nuclear protein binding sites of the TCR δ enhancer. These include three GATA binding sites (in δE1 and δE4), a κE3 like sequence (in δE3), an Ets binding site (in δE5), and a TCF–1/LEF–1 binding site (in dE7) (21, 69) (Figure 2B).

SHARED NUCLEAR PROTEIN BINDING SITES IN THE TCR PROMOTER/ENHANCERS

The coordinate induction of sets of tissue-specific genes is one of the hallmarks of mammalian development. The use of common sets of tissue-specific transcription factors to regulate multiple lineage-specific genes represents an economical mechanism for coordinating gene expression during development. Thus, it is not surprising that the TCR promoter/enhancers share overlapping sets of nuclear protein binding sites both with each other and with other T cell–specific genes (21). These sites and their consensus sequences are shown schematically in Figure 2 and Table 1. They include binding sites for the CREB/ATF family of leucine zipper transcription factors, the Ets family of protooncogenes, the GATA family of zinc finger transcription factors, the bHLH E box binding proteins, and the TCF–1/LEF–1 family of HMG transcription factors. In addition, there are several functionally significant shared binding sites for less well characterized nuclear proteins including Tα2B and CBF. Electrophoretic mobility shift assays have thus far demonstrated T cell–specific nuclear protein complexes that bind to the GATA and TCF–1/LEF–1 sites from several different T cell promoters and enhancers (72–80). The remainder of the sites appear to bind ubiquitously expressed or lymphoid-specific (in the case of the Ets sites) protein complexes. However, it remains possible that some of these complexes contain T cell–specific proteins or proteins that are post-translationally modified in a T cell–specific fashion. Thus far, none of the TCR promoter/enhancer nuclear protein binding complexes have been shown to be lineage-specific (i.e. restricted to α/β or γ/δ T cells). Although initially somewhat surprising, this finding is consistent with the observation that none of the previously described TCR promoters or enhancers display lineage-specific transcriptional regulatory activity in transient transfection assays.

Table 1 Transcription factors that regulate TCR gene expression

Transcription factor	Promoter enhancer binding sites	Molecular mass	DNA-binding domain	Recognition sequence	Lineage specificity	References
CREB		43 kDa	Basic domain/ leucine zipper		Ubiquitous	87, 88
CRE-BP1/ATF-2/ mXBP	α, β, Vβ	55 kDa	Basic domain/ leucine zipper	TGACGTCA	Ubiquitous	86, 89, 90
ATF-4		38 kDa	Basic domain/ leucine zipper		Ubiquitous	86, 91
TCF-1	α, β, δ	30 kDa	HMG Box	$C_{CT}C_TGAA$	T-cells	78, 79
TCF-1α/LEF-1		53–57 kDa	HMG Box	T T TTT	T-cells/ Pre-B-cells	78, 80
Ets-1	α, β, γ, δ	54–60 kDa	Basic domain/ α helix	$GA_{GGA}TG_CG$ CC AC	T-cells/B-cells	102
GATA-3	α, β, δ	47 kDa	2 Cx zinc fingers	$A_{GATA}A$ T G $CT_{GTGG}TTT$	T-cells/kidney/ brain	72–75
CBF	β, γ, δ	19–40 kDa	??	TC C CCA A	Thymus/Spleen	123

TRANSCRIPTION FACTORS THAT REGULATE TCR GENE EXPRESSION

During the last two years, several transcription factors that bind to the TCR α enhancer have been cloned and characterized. As described above, the TCR promoter/enhancers share binding sites for many of these proteins. Thus, these transcription factors each appear to be involved in regulating the expression of multiple TCR genes. However, because each of the cloned factors belongs to a family of related proteins, it remains unclear which family members are involved in regulating the expression of a given TCR gene at a specific point in T cell development.

CREB/ATF Proteins

Cyclic AMP response elements (CREs) are octanucleotide sequence motifs (TGACGTCA) which are present in the transcriptional regulatory regions of many eukaryotic genes (81–83). The name CRE is somewhat of a misnomer because these elements have been shown to mediate diverse transcriptional effects including (i) modulating transcription in response to alterations in intracellular cAMP (hence the name, CRE) (81, 82), (ii) conferring sensitivity to the adenovirus E1A gene product on certain adenovirus promoters (hence the name ATF) (83), (iii) conferring calcium responsiveness on certain promoters in neural cells (84), and (iv) modulating the basal levels of transcription of several mammalian genes (85). It is now clear that there is a large family of related proteins that can bind to CRE motifs (86–91). This CREB/ATF family of transcription factors consists of at least seven ubiquitously expressed members of the basic-leucine zipper family of transcription factors. These proteins share highly related basic DNA binding and adjacent leucine zipper dimerization domains but contain distinct N- and C-terminal sequences. Interestingly, both the structures of their DNA binding domains and their recognition sequences are highly related to those of the AP–1 family of transcription factors. Indeed, one recent report has demonstrated dimerization between one CREB/ATF family member (mXBP) and *c-jun*, a member of the AP–1 family (89). Given the large number of CREB/ATF family members, and the fact that these proteins can bind to DNA as both homodimers and heterodimers, the role of individual dimers in mediating the diverse effects ascribed to CREB proteins remains an important problem.

Functionally important CREs are found in both the TCR α and β enhancers (50), as well as in all human and murine Vβ promoters that have been characterized (50, 56, 57). Previous studies have shown that these sites bind at least five members of the CREB/ATF family of transcription factors from T cell nuclear extracts (50). Each of these nuclear protein

complexes appears to be expressed ubiquitously and, thus far, no T cell–specific CREB family member has been described. Southwestern screening has identified three CREB/ATF family members that bind to the CRE sites in the TCR α enhancer (91). The first clone corresponded to CREB, a 43-kDa transcription factor that has been shown to bind to CRE sites in several viral and cellular enhancers, and to confer cAMP-dependent transcriptional activation on these enhancers (87, 88, 92, 93). The second clone corresponded to a cDNA called CRE-BP1 (90), mXBP (89) or ATF–2 (86); this protein also binds with high affinity to CRE sites in several adenovirus promoters, as well as to the X box of murine MHC class II promoters. Binding of this protein to CRE sites in several adenovirus promoters confers responsiveness to the viral E1A protein (94). The final clone identified was CREB–2, a full-length homologue of a partial-length cDNA, previously called ATF–4 (86). The function of CREB–2/ATF–4 remains unknown; however, overexpression of CREB–2 in monkey CV–1 cells specifically downregulated cAMP-dependent transcription from the proenkephalin promoter/enhancer (91). Thus, at least in certain cell types, CREB–2 may represent a repressor of CRE-dependent transcription. The identities of the additional one to three CREB proteins that bind to the TCR α enhancer, and the function of each of the CREB proteins in regulating T cell transcription are unknown. However, a recent study detected a thymocyte-specific nuclear protein complex that binds to the CRE present in Vβ promoters (95). Interestingly, the kinetics of expression of this binding activity paralleled the rearrangement of TCR β genes in fetal thymocytes.

In summary, CREB/ATF proteins appear to play an important role in regulating TCR gene expression. There are multiple CREB proteins expressed in mature T cells, and there may be additional family members expressed in thymocytes. Ongoing studies are designed to identify all of the CREB/ATF family members expressed in T cells, to more precisely delineate their patterns of expression during T cell development and activation, and to elucidate their function by expressing *trans*-dominant inhibitors in transgenic mice and by using homologous recombination to eliminate their expression in mice. An additional area of interest concerns the role of these proteins in modulating T cell gene expression in response to specific signalling events including changes in intracellular cAMP and calcium.

The TCF–1/LEF–1 Family of Transcription Factors

Several reports have described the purification and cloning of two related transcription factors, TCF–1 and TCF–1α/LEF–1 that bind to a pyrimidine-rich sequence (PyCTTTG/T T/A T/A) from the 5′ end of Tα2 in

the human TCR α enhancer (76–80). There are additional binding sites for these proteins in the TCR β enhancer, the TCR δ enhancer, and the CD3ε enhancer (Figure 2). These proteins have masses of approximately 30 kDa (TCF–1) (79) and 53–57 kDa (TCF–1α/LEF–1) (76, 80) and contain highly related 80–90 amino acid DNA-binding domains which display significant homology to a region of the high mobility group (HMG) family of DNA-binding proteins. Whereas LEF–1 appears to be the murine homologue of TCF–1α, TCF–1 is encoded by a distinct gene (76, 79, 80). Studies of the expression patterns of these genes have demonstrated that LEF–1 is expressed during all stages of T cell development (in both α/β and γ/δ T cell lineages) (80, 96). Interestingly, LEF–1 expression was observed in prothymocytes that do not express CD3ε and have not undergone even partial TCR gene rearrangements. In addition, it is expressed in murine pre-B-cells (80, 96). In contrast, TCF–1 displays a distinct pattern of expression, with TCF–1 mRNA limited to prethymocytes (cells expressing CD3ε and displaying TCR gene rearrangement and expression) as well as more mature T cells (78, 96). Moreover, unlike LEF–1, TCF–1 is not expressed in cells of the B lineage (96). The different patterns of expression of TCF–1 and LEF–1 suggested that they play important but distinct roles in regulating T cell development.

Multiple forms of both the TCF–1 and TCF–1α/LEF–1 proteins appear to be generated by alternative splicing (77, 78). These include variants of TCF–1, containing or lacking a proline-rich domain NH_2-terminal to the HMG box, as well as multiple forms of the protein with distinct COOH-terminal regions, including one rich in serine and threonine residues. Similarly, two splice variants of TCF–1α have been described, which contain or lack a serine-threonine-rich domain, immediately NH_2-terminal to the HMG box. The DNA-binding activities and transcriptional *trans*-activation potentials of these alternatively spliced variants have not been studied in detail, but it seems likely that the activities of these transcription factors may be regulated at both the post-transcriptional and post-translational levels.

The mechanism by which TCF–1 and LEF–1 activate TCR gene expression remains unclear. Mutagenesis of the TCF–1 binding site in the minimal TCR α enhancer greatly decreased enhancer function (63, 76, 77). Moreover, overexpression of both TCF–1 and TCF–1α/LEF–1 in B-cells, HeLa cells or COS cells led to low level (3–20-fold) *trans*-activation of reporter constructs containing TCF–1 binding sites from the TCRα or CD3ε enhancers (77, 79, 80). *Trans*-activation by LEF–1 and TCF–1α appears to require the presence of additional nuclear protein-binding sites because overexpression of these factors cannot *trans*-activate a reporter

construct containing only multimerized TCF/LEF–1-binding sites upstream of a minimal promoter (79, 80). A recent study (97) has demonstrated that LEF–1 interacts predominantly with the minor groove of the DNA helix and is capable of bending DNA at a 130° angle following binding. This property which is reminiscent of the activities of the bacterial IHF protein may be involved either in facilitating protein-protein interactions within the TCR enhancers and/or in bringing the enhancer into close proximity with the V region promoters. Thus TCF–1 and LEF–1 may function predominantly to facilitate the assembly of nuclear protein complexes via their effects on DNA structure. However, they may also have direct transcriptional activating properties. Current studies are focused on better understanding the role of DNA bending in the function of these proteins as well as their role in T cell development using homologous recombination in ES cells to eliminate their expression in mice.

The ETS Family of Transcription Factors

Members of the Ets family of protooncogenes are sequence-specific transcription factors that recognize purine-rich sequences in the transcriptional regulatory elements of a large number of eukaryotic genes (98–103). Using a "Southwestern" screening approach, we were able to demonstrate that the protein product of the Ets–1 protooncogene binds specifically to a purine-rich sequence (GAGGATGTG) located at the 3′ end of Tα2 in the TCR α enhancer (102). Additional Ets binding sites are also present in the TCR β and δ enhancers (21). Binding of Ets–1 to the TCR α enhancer was especially intriguing because Ets–1 is known to be preferentially expressed in thymocytes and T cells (as well as B-cells) (104), and because its expression is developmentally regulated during thymic ontogeny, with a time course that is almost identical to that of the TCR α gene: both are first expressed on days 17 and 18 in the fetal mouse thymus (105). Thus, Ets–1 is a candidate trigger factor for expression of the TCR α gene. The importance of the Ets–1 binding site for TCR α enhancer function was demonstrated by experiments showing that mutations in the core Ets–1-binding site in Tα2 abolished the activity of the minimal TCR α enhancer in Jurkat cells (102). Thus far, however, overexpression of Ets–1 in B-cells and in nonlymphoid cells has not resulted in the *trans*-activation of the TCR α enhancer (I-C. Ho, J.M. Leiden, unpublished). This suggests that other T cell–specific factors are required for *trans* activation of the TCR enhancer by Ets–1 (for example TCF/LEF–1 or GATA–3) or that other ETS family members regulate the activity of the TCR α enhancer in T cells. Evidence from several systems suggests that Ets proteins usually require interactions with additional transcription factors to activate the

basal transcription machinery (103, 106, 107). Alternatively, the transcriptional activity of Ets–1 may require post-translational modification; Ets–1 is known to exist in both phosphorylated and nonphosphorylated forms in T cells (108). These possibilities are by no means mutually exclusive—the activity of Ets proteins may be regulated at multiple levels.

As is the case for the CREB/ATF proteins it is now clear that T cells express a large family of related Ets proteins. These include Ets–1, Ets–2, Fli–1, GABPα, Elf–1, Erg–1, and Erg–3 (106, 107, 109; J. Fang, J. Leiden, unpublished results). Each of the Ets family members contains a conserved DNA binding domain consisting of adjacent basic and α-helical regions (110). Despite this similarity in the structure of the Ets and CREB/ATF DNA binding domains, the Ets proteins bind to DNA as monomers unlike the CREB/ATF proteins which bind as dimers (110). Outside of their conserved DNA binding domains the different Ets proteins generally display distinct sequences.

Detailed studies of the DNA binding specificities of the different Ets family members demonstrate that the proteins can be divided into subfamilies based upon the structures of their DNA binding domains (110). Members of these subfamilies display subtly different DNA binding specificities, and they thereby bind to and regulate different genes in resting and activated T cells (110). For example Elf–1 binds to the sequence (AGGAAC/A C/T G) present in the enhancers of several activation-specific T cells genes such as the IL–2, IL–5, GM-CSF, and HIV–2 genes, but Elf–1 fails to bind to the Ets binding sites (AGGATGTG) in the human TCR α and β enhancers (102, 110). Conversely Ets–1 binds to the TCR α and β enhancers but fails to bind to the activation specific genes. These findings provide a molecular mechanism whereby different Ets family members expressed in the same cell can differentially regulate gene expression in response to distinct developmental and activational signals. As is the case for the CREB and TCF/LEF proteins, a complete understanding of the functions of each of the Ets family members awaits a more detailed analysis of their patterns of expression during T cell development, their different DNA binding specificities, their post-translational modifications, and the effects on murine T cell development of abolishing expression of individual Ets family members using homologous recombination in ES cells.

GATA–3

The GATA family of transcription factors is comprised of at least three related zinc finger proteins that bind to the sequence WGATAR (75, 111). Initial studies of the GATA transcription factors focused on GATA–1, the prototype family member which is expressed exclusively in erythroid

cells, megakaryocytes, mast cells, and their common progenitors (65, 112–115). GATA–1 is a 413 amino acid polypeptide containing an unusual zinc finger DNA binding domain. It binds to functionally important transcriptional regulatory motifs in the promoters and enhancers of several erythroid-specific genes including the α, β and γ globin genes. Recent experiments, in which GATA–1 gene expression was eliminated by homologous recombination in embryonic stem cells, demonstrated that GATA–1 is required for the development of the erythroid lineage in mice (116). Thus, GATA–1 belongs to the small family of lineage-specific determination genes.

Subsequent studies identified functionally important GATA binding motifs in the TCR α and δ enhancers (72–74). These findings, together with the finding that GATA–1 is not expressed in T cells, led to the cloning of a novel GATA family member, GATA–3 from chicken, mouse, and human T cell cDNA libraries (72–75). Human GATA–3 (hGATA–3) is a 47-kDa polypeptide containing two zinc fingers that are more than 90% identical to those of GATA–1 (72). In contrast, the remainder of the protein is less than 12% identical to GATA–1. Studies of the expression pattern of hGATA–3 showed that, within hematopoietic cells, its expression is restricted to the $\alpha\beta$ and $\gamma\delta$ T cell lineages (72, 117). Interestingly, a slightly larger GATA–3 mRNA has been detected in kidney and in some renal mesangial cell lines (J. Leiden, unpublished data). The relationship between the kidney and T cell GATA–3 mRNAs has not been established. Finally GATA–3 mRNA expression has also been detected by in situ hybridization in the embryonic central nervous system, the epidermis, and the adrenal medulla (117). The role of GATA–3 in the development of these diverse organs remains unclear.

Overexpression of hGATA–3 (as well as cGATA–3 or mGATA–3) in nonlymphoid cells can *trans*-activate the expression of reporter constructs containing multimerized GATA sites from Tα3 or from a related sequence from the δ TCR enhancer (δE4) upstream of a minimal promoter (72, 74). Thus, unlike the CREB/ATF, Ets, and TCF–1α/LEF–1 proteins, hGATA–3 can *trans*-activate transcription from its cognate binding site in the absence of additional nuclear protein binding sites.

mGATA–3 is expressed early in thymocyte ontogeny with mGATA–3 mRNA levels detectable in thymus from day 12.5 embryos (117). In myc-raf transformed murine thymocyte cell lines GATA–3 expression is not observed in $CD2^+$ $Thy1^+$ $CD3\gamma^+$ $CD4/CD8^-TCR^-$ prothymocytes, but is consistently observed in $CD3\gamma^+CD3\delta^+CD3\varepsilon^+$ prethymocytes which display partial rearrangements of their TCR genes as well as in $CD3/TCR^+$ mature thymocytes (117). This pattern of GATA–3 expression parallels that of TCF–1. Thus, like TCF–1 and TCF–1α/LEF–1, GATA–3 is a

lineage-restricted transcription factor that is expressed very early in T cell development. hGATA–3 is also expressed at low levels in CEM cells, an immature T cell line that does not express a TCR mRNA. However, after stimulation with PMA, both hGATA–3 and TCRα mRNA levels increase dramatically (similar findings have been reported for TCF–1α in this system) (72). These findings, when considered with the previously described results concerning the role of GATA–1 in erythroid differentiation, suggest that GATA–3 is a good candidate for a T cell–determining gene. This possibility is currently being tested by using homologous recombination to eliminate GATA–3 expression in ES cells and mice.

Core Binding Factor

The transcriptional enhancers of the replication-competent murine leukemia viruses play an important role in determining the thymic tropism and T cell transformation potential of these viruses (118, 119). Several nuclear protein binding sites have been shown to be important in determining enhancer activity in T cells (120, 121). Among these sites is the Moloney core motif (CTGTGGTAA) (121) which has been highly conserved in several murine leukemia virus enhancers and has also been identified in the SV40 and polyomavirus enhancers. Mutation of the core motif of the Moloney virus specifically decreases transcriptional activity in hematopoietic cell lines and, more importantly, causes a significant change in the lineage-specificity of transformation of the virus in vivo (122). Wild type Moloney virus generally causes T cell lymphomas and leukemias in mice. In contrast, the virus containing core motif mutations causes erythroleukemias in more than 50% of the animals tested. The Moloney core motif has been shown to bind several nuclear protein complexes from T cell nuclear extracts. Recently Speck and coworkers (123) have reported the purification of core motif binding factors (CBFs) from calf thymus nuclei. SDS-PAGE analysis of these proteins revealed at least ten polypeptides ranging in molecular weight from 19,000–40,000. Because several of these polypeptides were highly related at the amino acid sequence level, it was postulated that they represented proteolytic cleavage products of one or several bona fide full-length CBFs. Interestingly CBF DNA binding activity was detected in thymus and spleen but not in liver, lung, or heart.

There are several reasons to think that CBF plays an important role in regulating TCR gene expression. First, potential core binding motifs are present in nuclear protein binding sites from the TCR β, γ, and δ enhancers (Tβ3, NFγ3 and NFγ4, and δE3, respectively) (Figure 2). Secondly, two of these sites (Tβ3, and δE3) have been shown by mutagenesis analysis to

be important for enhancer function (50) (M. Krangel, personal communication). Finally, Wang & Speck (123) have directly demonstrated binding of purified CBF to NFγ3 and NFγ4 from the murine TCR γ enhancer. A better understanding of the role of CBF(s) in regulating TCR gene expression awaits cloning of the cDNA(s) encoding this factor and an analysis of its DNA binding potential, transcriptional activity, and developmental regulation in lymphoid and nonlymphoid cells.

SUMMARY AND FUTURE DIRECTIONS

During the last several years, we have learned a great deal about the molecular mechanisms that regulate TCR gene expression during thymocyte development. Many of the important *cis*-acting transcriptional activator sequences have been identified and characterized, and a set of novel transcription factors, some of which are T cell–specific, have been cloned. Several of these factors, including the CREB, TCF–1, TCF–1α LEF–1, Ets, CBF, and GATA–3 proteins appear to play important roles in regulating the expression of multiple genes during T cell development and activation. Because they are T cell–specific and expressed very early during thymocyte ontogeny, the TCF–1, TCF–1α LEF–1, and GATA–3 proteins are particularly good candidates for T cell determining genes.

Nevertheless, a number of important questions remain. First, detailed mutational and deletional analyses of the TCR γ and δ enhancers have not yet been reported. Secondly, several of the transcription factors that bind to the TCR enhancers have not yet been fully characterized and cloned. For example the identities of the Tα2B, Tα4, CBF, and several of the TCR δ and γ enhancer binding proteins remain unclear. Moreover, it is not yet known which E box-binding proteins are expressed in T cells. Given the importance of this family of transcription factors (the bHLH proteins such as E12/E47 and MyoD) in other developmental systems, this remains an important issue. In addition, because many of the TCR enhancer binding proteins belong to families of transcription factors, it remains unclear which of the family members is involved in regulating a given TCR gene at a specific point in T cell development. Finally, the available evidence suggests that many of these transcription factors may themselves be regulated at the transcriptional, post-transcriptional, and post-translational levels, and we currently understand relatively little about these processes or their significance in regulating T cell development.

One of the more surprising findings that has come out of the recent work on TCR gene regulation is the fact that although each of the TCR enhancers is T cell–specific, none display transcriptional activity that is

restricted to the TCR α/β or TCR γ/δ lineages. There are three possible explanations that can reconcile this finding with the fact that the rearrangement and expression of at least several of the TCR genes is lineage-specific. First, the lineage-specific expression of these genes may be controlled not by transcription but by lineage-specific rearrangements, which in turn are controlled by non-enhancer dependent mechanisms. This possibility seems especially attractive in the case of the TCR δ gene which is deleted on both alleles in most α/β T cells. Second, it is possible that there are additional as yet unidentified lineage-specific enhancers in the TCR loci. This seems unlikely because the previously identified enhancers are quite potent, and because most of the relevant portions of the TCR loci have been screened for enhancer activity. Third, the previously identified enhancers may be lineage-specific in vivo but may lack lineage specificity in the transient transfection assays used in most previous studies. Finally, it is possible that lineage-specific negative transcriptional regulatory sequences or silencers are important in restricting the expression of the TCR genes. This possibility seems likely given the recent identification of such negative regulatory elements in the TCR α and γ genes. Future studies designed to localize and characterize these silencers more precisely should help to elucidate their role in regulating TCR gene expression in vitro and in vivo.

Over the next several years, all of the transcription factors involved in regulating TCR gene expression should be identified and cloned. Gene knock-out experiments, using homologous recombination along with the expression of *trans*-dominant inhibitors in vitro and in vivo, should help to shed light on the function of individual transcription factors in regulating TCR gene expression, and the more general process of T cell development. Complementary transgenic experiments using different deleted and mutated forms of the TCR enhancers and silencers should help us to understand the roles of these different elements in controlling the lineage-specific rearrangement and expression of these genes. Biochemical studies should help to elucidate the post-transcriptional and post-translational regulation of these factors. Finally, studies of the transcriptional regulation of these transcription factors themselves may move us one step closer to identifying the bona fide T cell–determining gene(s). Given the current level of interest in these problems and the reagents now available, such advances in our knowledge can be expected in the near future.

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THE MOLECULAR CELL BIOLOGY OF INTERFERON-γ AND ITS RECEPTOR

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Abstract

The last ten years have seen an explosive growth in our understanding of IFNγ. The cloning of the cDNAs for IFNγ and its receptor have made available large amounts of highly purified recombinant IFNγ and soluble IFNγ receptor. In addition, highly specific neutralizing monoclonal antibodies have been generated to both of these proteins. Using these reagents, IFNγ and the IFNγ receptor have been characterized on a molecular basis. Structure-function studies carried out on these proteins have identified key molecular regions that are required for biologic activity. Moreover, a great deal is now known concerning the physiologic role that IFNγ plays in vivo. In this review we focus on the new developments in the areas of IFNγ biochemistry and biology and pay particular attention to the IFNγ receptor and three aspects of IFNγ biology that are of special interest to immunologists: host defense, inflammation, and autoimmunity.

INTRODUCTION

Interferon-gamma (IFNγ) was first recognized nearly 30 years ago on the basis of its antiviral activity (1). During the ensuing years, a great deal of information has accumulated which unequivocally establishes that this pleotropic cytokine plays an important role in modulating nearly all phases of immune and inflammatory responses. During the past 10 years, the genes for IFNγ and its receptor have been cloned, and the structures of

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the corresponding proteins determined. Large amounts of highly purified recombinant IFNγ and IFNγ receptor are now available as well as neutralizing monoclonal antibodies specific for either the cytokine or its receptor. The availability of these reagents has facilitated many studies aimed at elucidating IFNγ's mechanism of action at the molecular level and defining its physiologic role in vivo. Ironically, the information derived from 30 years of IFNγ research now points to the relatively minor physiologic importance that IFNγ has as a direct antiviral agent. In the current review we focus on some of the more recent developments in the areas of IFNγ biochemistry and biology, and we place particular emphasis on work concerning the IFNγ receptor.

THE RELATIONSHIP BETWEEN IFNγ AND OTHER INTERFERONS

IFNγ belongs to a family of proteins related by their ability to protect cells from viral infection. Based on several criteria, the interferons have been divided into three distinct classes termed interferon-α, -β, and -γ (Table 1). IFNα (originally known as Type I IFN or Leukocyte IFN because it was produced by peripheral blood mononuclear cells) and IFNβ (also originally known as Type I IFN or Fibroblast IFN because of its cell of origin) are classical interferons induced in response to viral infection of cells (2, 3). Twenty six IFNα genes (including several pseudogenes) have been identified (4). The genes have common structures, i.e. they lack introns and appear to derive from a common ancestral precursor (5). The IFNα gene cluster resides on human chromosome 9 and murine chromosome 4 (6). This gene family encodes at least 22 distinct proteins which are single polypeptide chains of 165–166 amino acids with molecular weights of approximately 20 kDa (7). The reasons for the complexity of the IFNα gene system remain unclear, but recent studies suggest that the different IFNα species effect distinct arrays of biological responses in different cells (7). There is only a single form of IFNβ. It is encoded by a distinct gene located next to the IFNα locus in both human and mouse. Based on the general organization of their genes and proteins, IFNα and IFNβ are thought to have evolved from a common ancestral precursor. However, IFNβ shares only limited antigenic relatedness to the IFNα family, and the proteins display only 15–30% amino acid sequence homology (3). Nevertheless, both forms of Type I IFN bind to the same receptor on the surface of susceptible target cells, indicating that at least some of the functionally important regions of the molecules have been conserved. Although an earlier report suggested the existence of a second IFNβ species, designated IFNβ2 (8), subsequent studies revealed that the

Table 1 Properties of the interferons

Property	IFNα	IFNβ	IFNγ
NOMENCLATURE	Type I Leukocyte	Type I Fibroblast	Type II Immune
MAJOR INDUCERS	Virus	Virus, LPS ds-poly RNA	Antigens Mitogens
PHYSICAL PROPERTIES			
M.W. (kDa)			
Predicted/mature	20/20	20/20–25	17/34–50
Amino acids	165–166	166	143
N-linked glycosylation	Some species	+	2 sites
Subunit composition	Single polypeptide	Single polypeptide	Noncovalent homodimer
pH stability	Stable	Stable	Labile
GENE STRUCTURE			
Number of genes	26	1	1
Chromosomal location			
Murine	4	4	10
Human	9	9	12
Presence of introns	None	None	3
CELLULAR SOURCE	T cells, B cells and macrophages	Fibroblasts and epithelial cells	T cells and NK cells
CELLULAR RECEPTOR			
M.W. (kDa)			
predicted/mature	60.5/95–100		52.6/85–95
Amino acids	530		472
Domain structure			
Extracellular	409 a.a.		228 a.a.
Transmembrane	21 a.a.		23 a.a.
Intracellular	100 a.a.		221 a.a.
Glycosylation sites	12		5
Chromosomal location			
Murine	16	16	10
Human	21	21	6

observed activity was ascribable to IL–6 which elicited antiviral responses in certain in vitro assays in an indirect manner (9, 10)

IFNγ (also termed Type II IFN or Immune IFN) is unrelated to the Type I interferons at both the genetic and the protein levels (11–13). Moreover, IFNγ is induced by a unique set of stimuli and is produced only by T lymphocytes and natural killer (NK) cells. Importantly, viral infection of these cells does not directly induce IFNγ production. Although IFNγ displays most of the biologic activities that have been ascribed to the other interferons, it has a 10–100 fold lower specific antiviral activity than either

IFNα or IFNβ. On the other hand, IFNγ is 100–10,000 times more active as an immunomodulator than are other classes of interferons (14). This observation has lead to the concept that whereas IFNα/β are primarily antiviral agents which display some immunomodulatory activity, IFNγ is primarily an immunomodulator that also can exert some antiviral activity (15).

MOLECULAR CHARACTERISTICS OF HUMAN AND MURINE IFNγ

The cDNAs for human and murine IFNγ were first cloned in 1982 by Gray & Goeddel (11–13), and today a great deal is known about the structure of the IFNγ genes and the corresponding proteins they encode. Both humans and mice contain only a single IFNγ gene. The IFNγ gene is considerably more complex than the genes for either IFNα or IFNβ. The human and murine genes are 6 kb in size, and each contains four exons and three introns. Using in situ hybridization techniques, the genes for human and murine IFNγ have been localized to human chromosome 12 (12q24.1) and murine chromosome 10, respectively (6, 16, 17). The regulatory elements in the IFNγ gene have been only partially characterized. A tissue-nonspecific enhancer element has been detected in a 220 bp sequence within the first intron of the human gene (18). Positive and negative *cis*-acting promoter elements have been identified in a region 700 bp immediately 5′ to the transcription start site (18, 19). The sequences in the promoter responsible for tissue-specific gene expression remain unknown. Interestingly, when DNA containing the entire human IFNγ gene and 2.3 and 1.0 kb of 5′ and 3′ flanking genomic sequence, respectively, was introduced into the murine germ line, the resulting transgenic mice displayed inducible expression of human IFNγ in a tissue appropriate manner (20). In contrast, when the human gene was introduced into a variety of different cultured murine cell lines (such as T cells and fibroblasts), the tissue specificity of expression was lost (21, 22). Thus, it has been suggested that regulation of IFNγ gene expression must also depend on as yet undefined developmental factors.

Activation of the human gene leads to the generation of a 1.2 kb mRNA that encodes a 166 amino acid polypeptide (11, 23). The amino terminal 23 residues of the human protein constitute a typical hydrophobic signal sequence which, when proteolytically removed, gives rise to a mature 143 residue positively charged polypeptide with a predicted molecular mass of 17 kDa (Figure 1). Although the amino terminus of the mature polypeptide was originally predicted to be Cys-Tyr-Cys (11), amino terminal sequence analysis performed on natural forms of human IFNγ demonstrated that

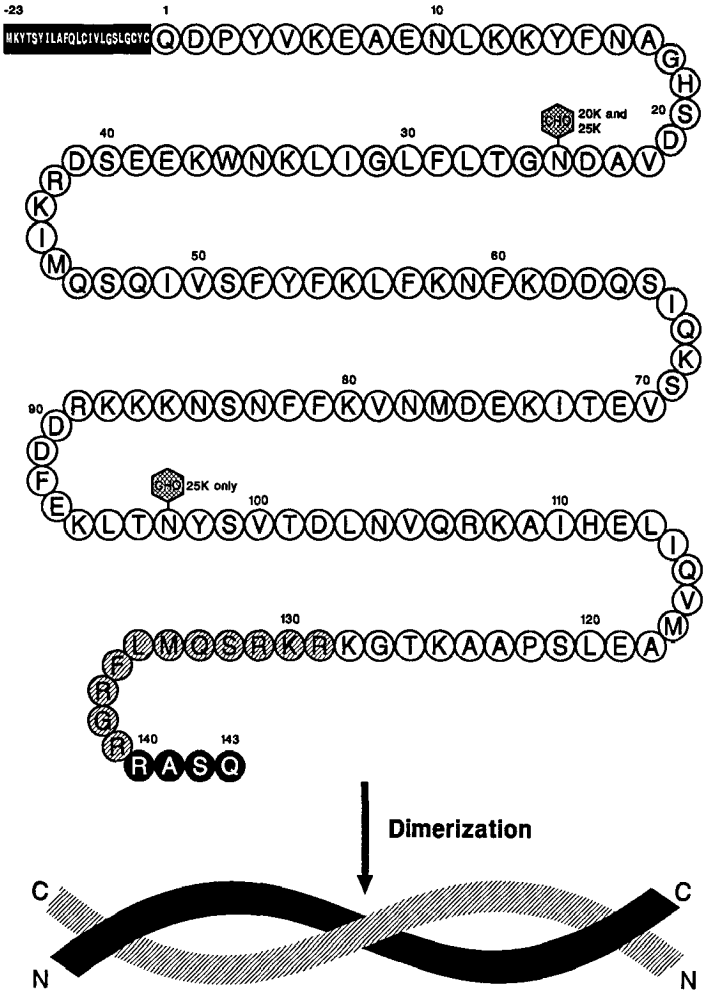


Figure 1 Schematic representation of the human IFNγ molecule. IFNγ is synthesized as a 166 amino acid polypeptide that contains a 23 amino acid signal sequence (designated in the shaded box). The mature protein contains 143 amino acids. The location of the two N-linked glycosylation sites is noted. A functionally important region of the molecule has been mapped to the amino terminal 39 residues. In addition, a second important region has been localized to the carboxy terminal 15 residues. Two IFNγ polypeptides self-associate to form a noncovalent homodimer.

the amino terminus was actually pyroglutamic acid (Figure 1) (24). The carboxy terminus of the molecule is susceptible to post-translational enzymatic degradation (24). At least six different carboxy termini have been detected on natural and recombinant forms of human IFNγ. Since this portion of the molecule contains a large number of positively charged residues, the various truncations contribute to the charge heterogeneity seen in the fully mature molecule. Two polypeptides self-associate to form a homodimer with an apparent molecular weight of 34 kDa (25–30). At physiologic concentrations, little if any monomer is detectable. Only the dimer can display IFNγ biologic activity, possibly because it is the only form of the molecule that can effect IFNγ receptor dimerization (31, 31a, 32). Since the mature human IFNγ polypeptide is devoid of cysteine, the homodimer is held together entirely by noncovalent forces. The quaternary structure of the molecule explains its characteristic sensitivity to extremes of heat (the protein is denatured at temperatures above 56°C), and pH (activity is rapidly lost at pH values less than 4.0 and greater than 9.0) (33–37)

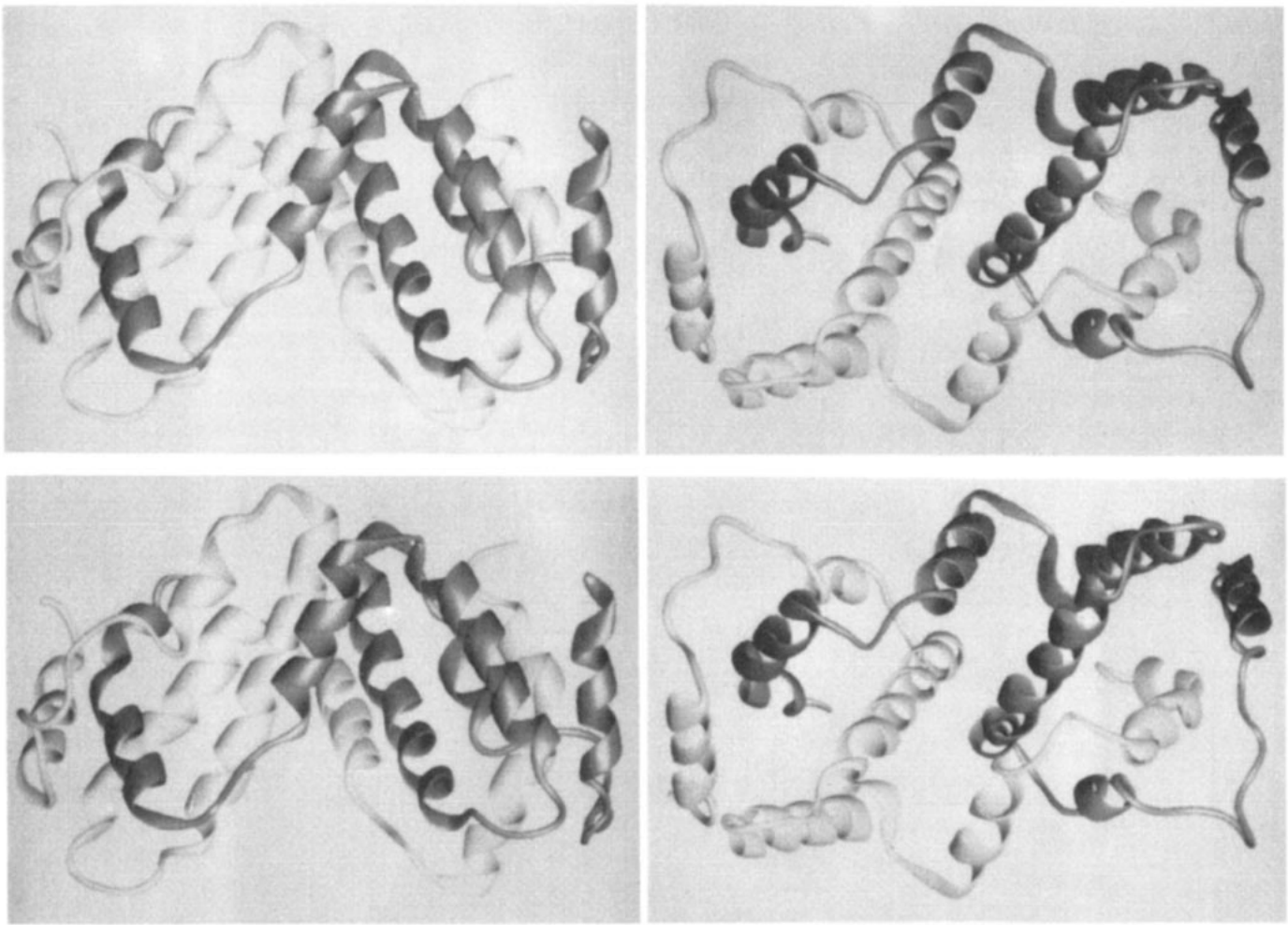
The murine gene gives rise to a 1.2 kb mRNA that encodes a mature 134 amino acid polypeptide with a predicted molecular mass of 15.4 kDa (13). Like its human counterpart, murine IFNγ exists exclusively as a noncovalent homodimer. Human and murine IFNγ display only modest homology at either the cDNA or amino acid levels (60% and 40%, respectively). This low level of sequence homology explains why the human and murine proteins display a strict species specificity in their ability to bind to and activate human and murine cells.

The individual human and murine polypeptide chains contain two N-linked glycosylation sites (residues 25 and 97 in mature human IFNγ and residues 16 and 69 in the mature form of murine IFNγ) that are independently and differentially glycosylated, thereby giving rise to subunits of differing molecular weights. Natural human IFNγ is composed of polypeptides that display molecular masses of 17, 20, and 25 kDa, which correspond to molecules with 0, 1, or both glycosylation sites occupied (38, 39). This differential glycosylation accounts for much of the observed molecular weight heterogeneity in the fully mature homodimeric molecules (i.e. natural human and murine IFNγ display molecular weights that range from 30–50 kDa). Whereas glycosylation is not important for expression of IFNγ activity, it appears to influence the circulatory half-life of the molecule (38, 40, 41).

The functionally important regions of the IFNγ molecule are now being elucidated. Current data indicate that both the amino and carboxy terminal regions play critical roles in maintaining an active conformation of the protein. The importance of the amino terminal region has been docu-

mented by several independent studies. Monoclonal antibodies specific for amino terminal portions of human and murine IFNγ block binding of IFNγ to cellular receptors and neutralize IFNγ's ability to induce antiviral activity in fibroblasts and activate nonspecific cytocidal activity in macrophages (42–46). In addition, a synthetic peptide with a sequence that corresponds to the amino terminal 39 amino acids of human IFNγ has been shown to block binding of IFNγ to cell surfaces when supplied in high concentration (44, 45). Finally, human and murine IFNγ molecules lacking the first 10 amino terminal residues (produced either by enzymatic digestion of the molecule with *Staphylococcus aureus* V–8 protease—47, 48—or using molecular genetic approaches—49, 49a) are devoid of biologic activity. Other studies have provided evidence for an important role of the carboxy terminal portion of the molecule. Although removal of the carboxy terminal 9 amino acids from human IFNγ results in little or no reduction in biologic activity, enzymatic removal of residues 129–143 of the protein with various endoproteases (such as clostripain or submaxillaris protease—50, trypsin or pronase—28, 50), results in a 10–100 fold reduction in IFNγ's specific activity (as determined by monitoring IFNγ-dependent induction of antiviral activity in fibroblasts and Fc receptor expression on mononuclear phagocytes). In addition, the enzymatically truncated form of IFNγ displays a 1000-fold reduction in receptor binding affinity (50). A truncated form of human IFNγ, generated by placing a stop codon after residue 125, also showed a similar reduction in the capacity to bind to IFNγ receptors and induce IFNγ-dependent cellular responses (51, 52). Finally, certain monoclonal antibodies reactive with carboxy terminal peptides of human (53) and murine (54) IFNγ neutralize the protein's antiviral activity.

Recently, the x-ray crystallographic structure of human IFNγ was solved to 3.5 Å (55) (Figure 2). This study confirmed the dimeric nature of the mature protein. The individual subunits have a flattened prolate elliptical shape. However, the overall structure of the dimer is compact and globular. The molecule appears to be primarily helical (62%) and lacks β sheet structure. Each subunit consists of six α helices held together by short nonhelical regions. The dimer is formed by a unique intertwining of the helices across the subunit face which provides an opportunity for multiple interactions along each subunit. This type of intimate subunit interaction is extremely unusual and has been seen in only a few other proteins. The model predicts that the subunits associate in an antiparallel fashion, thereby leading to a juxtaposition of the amino and carboxy terminal portions of the opposing polypeptide chains. Whereas the amino termini coordinates have been firmly established, the carboxy termini do not adopt a rigid conformation in solution, as determined using either x-



ray crystallographic or nuclear magnetic resonance approaches (56, 56a). Nevertheless, the model suggests that each IFNγ dimer may be able to bind two IFNγ receptors. Experimental data recently obtained support this possibility (31, 31a, 32)

IFNγ BIOSYNTHESIS

In the normal host, the T lymphocyte represents the major cellular source of IFNγ. All $CD8^+$ T cell populations and certain subsets of $CD4^+$ T cells can produce the protein (57, 58) (Figure 3). IFNγ synthesis has been demonstrated in the T_H1 helper T cell subset, and data has also been obtained indicating that it may be produced by a less differentiated/activated type of $CD4^+$ T cell designated T_H0 (59–61). The external stimuli that induce IFNγ production by T cells are similar to those that induce other T cell–derived cytokines (57, 58, 62). The primary physiologic stimulus is antigen in the context of either major histocompatibility (MHC) class II (for $CD4^+$ T cells) or MHC class I antigens (for $CD8^+$ T cells). Experimentally, IFNγ can also be induced by either (1) direct stimulation of the T cell receptor/CD3 complex with antibodies such as anti-CD3, (2) T cell mitogens (such as concanavalin A or phytohemagglutinin) or (3) pharmacologic stimuli (such as the combination of phorbol myristate acetate and calcium ionophore) (63). In addition, T cell–dependent production of IFNγ is enhanced by products of activated T cells and macrophages such as IL–2, hydrogen peroxide, and leukotrienes LTB4, LTC4, and LTD4 (64–69). Stimulation of T cells results in the induction of IFNγ mRNA which is first detectable at 6–8 hours, peaks by 12–24 hours, and slowly declines thereafter. The protein is secreted immediately after synthesis. It can first be detected in the extracellular environment 8–12 hours after stimulation and reaches peak levels after 18–24 hours. IFNγ produced as a result of experimental in vitro T cell stimulation (such as during the mixed leukocyte reaction) is not significantly consumed by the cells of the culture and therefore can be detected in the medium long after the T cell activation response has ended (70). In contrast, IFNγ is rarely seen in the circulation of humans or mice undergoing immunologic stimulation. This apparent discrepancy between in vitro and in vivo levels of

Figure 2 Stereo views of the human IFNγ homodimer as derived from coordinates obtained from the x-ray crystallographic analysis of the protein. The crystal structure has been resolved to 3.5 angstroms. The ribbon drawings are based on the Cα positions. Panel A: the view is approximately parallel to the dimer two-fold axis. Panel B: The view is approximately perpendicular to the dimer two-fold axis. The precise position of the carboxy terminus has not been established. (Reprinted with permission from Ealick et al (55).)

fluid phase IFNγ is most likely due to the rapid removal of IFNγ from the circulation by IFNγ receptors that are ubiquitously expressed on nearly all cells (71)

Two newly described cytokines are noteworthy in their respective abilities to regulate IFNγ production in either a positive or negative manner. IL–12 (formerly called NK stimulatory factor, NKSF) is a product of B cells and macrophages and induces IFNγ gene expression in T cells and NK cells in a manner that is at least partially distinct from the conventional pathway of T cell activation (72, 73). IL–12 dependent IFNγ induction is insensitive to cyclosporin A and is synergistic with phytohemagglutinin, phorbol esters, anti-CD3, IL–2, and allogeneic antigens but not Ca^{++} ionophores (74). The precise stimuli that lead to IL–12 induction in vivo are not yet characterized. On the other hand, another newly described cytokine, IL–10, *inhibits* IFNγ production by T cells (75, 76). The inhibitory effects of IL–10 on IFNγ production are more profound than effects on the production of other T_H1 cytokines such as IL–2. IL–10 is produced by the T_H2 $CD4^+$ T cell subset, as well as B cells and macrophages (77). The mechanism by which IL–10 exerts its inhibitory effects on IFNγ

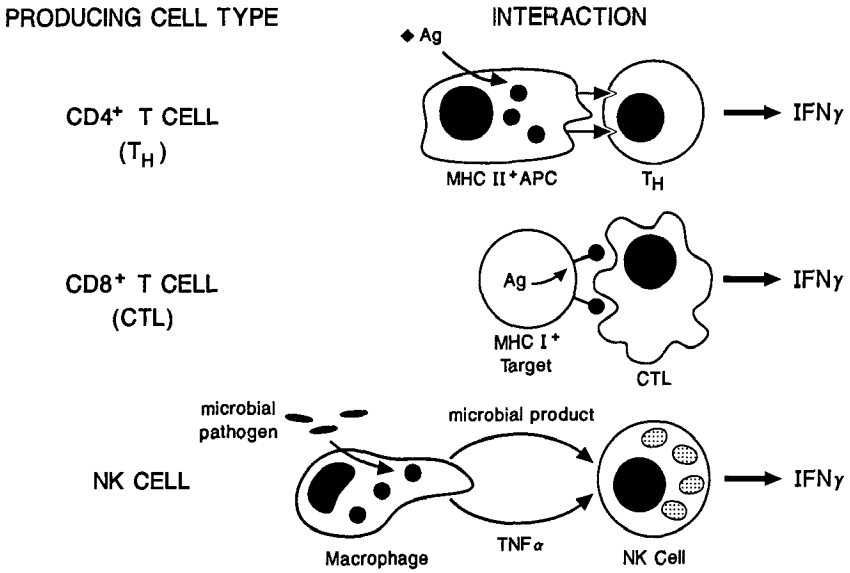


Figure 3 Cellular sources of IFNγ. IFNγ can be produced either by $CD4^+$ T cells in response to antigen presented in the context of MHC class II molecules or by cytotoxic T lymphocytes following recognition of antigen associated with MHC class I. In addition, NK cells elaborate IFNγ after exposure to TNFα and microbial products.

production is only now being elucidated. However, the target of IL–10 action clearly is the antigen presenting cell and not the T cell (78). The current hypothesis is that IL–10 may inhibit the expression of APC-derived accessory molecules required to induce full activation of the T cell for IFNγ production.

More recent studies have demonstrated that IFNγ can also be produced by natural killer cells (NK) (79–83). Mitogens can induce IFNγ from populations of human and murine NK cells expanded in vitro with IL–2. However, of far greater importance is the observation that bacteria and/or microbial products can rapidly stimulate IFNγ production from naive NK cell populations either in vitro or in vivo (80, 81, 83). This conclusion is based on experiments performed using either normal mice or immunodeficient C.B–17 mice expressing the scid mutation (SCID). SCID mice lack the ability to generate rearranged T cell receptor and immunoglobulin genes and therefore are completely devoid of functional T and B cells. Initial experiments indicated that SCID mice were capable of elaborating activated, MHC class II–positive macrophages during infection with *Listeria monocytogenes*, and they displayed partial resistance to the infection, responses that have been shown to be obligatorily dependent on IFNγ. The ability of SCID mice to produce IFNγ was unequivocally established using a neutralizing monoclonal antibody specific for murine IFNγ. SCID mice pretreated in vivo with anti-IFNγ failed to elaborate activated, MHC class II–positive macrophages, and they died following injection with a sublethal dose of live *Listeria*. In addition, IFNγ could be immunochemically identified in culture supernatants of SCID splenocytes stimulated in vitro with heat killed *Listeria* (HKLM). Treatment of SCID splenocytes with anti-asialo GM1 and complement ablated their ability to produce IFNγ when exposed to HKLM, thereby indicating that the IFNγ producing cell was an NK cell (81, 84).

Subsequent studies identified TNFα and macrophages as two of the cofactors required for the generation of IFNγ by NK cells (81–83). In vitro stimulation of SCID splenocytes with HKLM was found to effect production of *both* TNFα and IFNγ but with different kinetics. Peak levels of TNFα were observed 18 hr after stimulation and always preceded production of IFNγ which peaked only after 48 hr. Whereas the addition of neutralizing monoclonal antibody to TNFα prevented elaboration of IFNγ, control antibodies (including neutralizing antibodies specific for murine IL–1) did not. Moreover, injection of neutralizing anti-TNFα into either SCID or normal mice blocked the ability of the animals to produce IFNγ in vivo and to mount an anti-*Listeria* response. Macrophages from antibody-treated, infected mice did not show increased levels of MHC class II, and the mice failed to clear the bacteria from the spleen and liver

(81). These results thus show that generation of IFNγ by stimulated SCID spleen cells required the production of TNFα.

Cell depletion experiments revealed that macrophages were the cellular source of TNFα. Moreover, cell mixing experiments using pure populations of SCID NK cells derived by in vitro propagation of cells in IL–2 showed that IFNγ production occurred only in the presence of macrophages and bacteria or in the presence of purified TNFα and soluble bacterial products (82, 83). In the latter case, TNFα alone was not sufficient to induce IFNγ production from NK cells. Thus, NK cell activation to produce IFNγ requires two components: TNFα produced physiologically upon exposure of a macrophage to a microbial pathogen and a second stimulus that can be a bacterial product. Taken together, these results indicate that the IFNγ produced by NK cells represents the host's first line of defense against microbial pathogens that are susceptible to killing by activated macrophages.

THE IFNγ RECEPTOR AND MECHANISMS OF SIGNAL TRANSDUCTION

IFNγ exerts its pleiotropic effects on cells through an interaction with a specific receptor expressed at the cell surface. On the basis of immunochemical, radioligand binding, and molecular genetic analyses, there appears to be only a single type of IFNγ receptor that is ubiquitously expressed on all cells (except the erythrocyte) (85, 103, 86–95). Even platelets express IFNγ receptors at a level of 300 receptors/cell (96). Considering the large number of platelets in the circulation (3×10^8/ml), it is possible that this cell plays an important role in transporting IFNγ through the circulatory system. It is noteworthy that when receptor expression in different tissues is analyzed at either the mRNA or protein levels, the highest expression is observed in tissues not generally considered to have primary immunologic functions (95; M. Luquette, J. Calderon, R. D. Schreiber, unpublished results). Specifically, skin, nerve, and syncytial trophoblasts of the placenta express levels of IFNγ receptor that are often 10–100 times that observed in spleen or on hematopoietic cells. The receptor binds ligand with high affinity ($Ka = 10^9 - 10^{10}\ M^{-1}$) and is expressed on most cells only at modest levels (200–25,000 sites/cell). Human and murine IFNγ receptors display strict species specificity in their ability to interact with human and murine IFNγ.

IFNγ RECEPTOR SYNTHESIS AND RECYCLING

Using radioligand binding and immunoprecipitation techniques, the lifecycle of human and murine IFNγ receptors have been partially elucidated

(97–100). The receptor is synthesized in the endoplasmic reticulum and is glycosylated as it moves from the ER through the golgi. At least three glycosylation intermediates with molecular masses of 65, 70, and 75 kDa have been identified. Only fully glycosylated receptors are expressed at the cell surface. Carbohydrate analysis indicates that most if not all of the carbohydrates are N-linked. However, O-linked glycosylation of receptors in some cells has not been stringently ruled out. Fully mature receptors expressed on the plasma membrane display molecular masses that vary between 80 and 95 kDa. This modest molecular weight heterogeneity is a result of cell-specific differences in glycosylation. Following interaction with ligand at the cell surface, the intracellular domain of the receptor is phosphorylated on serine and threonine residues (101, 102). Although the functional significance of this phosphorylation remains unclear, the rate and extent of phosphorylation correlates perfectly with the magnitude of the biologic response induced. The phosphorylated receptor-ligand complex is internalized, enters an acidified endosomal compartment, and dissociates. Free IFNγ eventually traffics to the lysosome where it is ultimately degraded. In many cells, such as fibroblasts, the uncoupled receptor enters a large intracellular pool of mature receptor and eventually recycles back to the cell surface. The size of the intracellular receptor pool is generally 2–4 times greater than the pool of receptors expressed at the cell surface (103–105). The ability of the IFNγ receptor to recycle on macrophages remains controversial. Whereas some reports indicate that recycling does indeed occur on either primary or cultured mononuclear phagocytes (97, 103, 105), at least one other group has reported that internalized receptors are degraded on primary monocytes (106).

Receptor Structure

Recently, human and murine IFNγ receptors have been purified to homogeneity (89–92) and partially characterized. The complete characterization of the human receptor structure was made possible by the cloning of its cDNA, first accomplished by Aguet and colleagues in 1988 (107). Subsequently, several laboratories cloned and expressed the murine homologue (108–112). The genes for the human and murine receptors have been localized to chromosomes 6 (q16-q22) and 10, respectively (113–115). The human and murine IFNγ receptor genes are approximately 30 kb in size (116). Each consists of 7 exons and, upon activation, gives rise to a single 2.3 kb mRNA transcript. The resulting human and murine proteins are organized in a similar manner (Table 2, Figure 4). The mature proteins consist of 472 and 451 amino acids, respectively, and have predicted molecular masses of 52.5 and 48.5 kDa. Both proteins are symmetrically oriented around single 23 amino acid transmembrane domains. Each pos-

Table 2 Comparison of human and murine IFNγ receptors

Property	IFNγ receptor	
	Human	Murine
Primary sequence		
Signal peptide	17aa	26aa
Mature form	472aa	451aa
Homology	52%	
Domain structure		
Extracellular	228aa	228aa
Transmembrane	23aa	23aa
Intracellular	221aa	200aa
Potential N-linked Glycosylation sites	5	5
Predicted molecular weight	52,563	49,819
Molecular weight mature molecule	90,000	90,000
Serine/threonine content		
Whole molecule	17.6%	20.0%
Extracellular domain	11.8%	15.8%
Intracellular domain	23.3%	24.6%

sesses a 228 amino acid extracellular domain that contains 10 cysteine residues and 5 potential N-linked glycosylation sites. Based on biosynthetic labeling experiments, all 5 glycosylation sites appear to be occupied (98, 99, 117), and N-linked oligosaccharides contribute approximately 25 kDa to the apparent molecular weight of the fully mature protein. Soluble forms of the extracellular domain of the human and murine IFNγ receptors have been produced either in bacteria or eukaryotic cells (31, 118, 119). The availability of these reagents has led to the demonstration that the extracellular domain is sufficient to account for high affinity ligand binding and that receptor glycosylation is not critical for ligand binding activity. A structure-function analysis of this domain has been initiated. Proteolytic digestion of the receptor's soluble extracellular domain demonstrated that the amino terminal six residues are not required for ligand binding activity. However, the smallest proteolytic fragment of the *E. coli*–derived soluble receptor capable of expressing full ligand binding activity was a 25 kDa component encompassing residues 6–227, which is nearly the entire extracellular domain (120). Obviously, more work is needed before the structural elements of the receptor that are involved in ligand binding are defined.

Based on primary sequence comparisons, the IFNγ receptor bears little

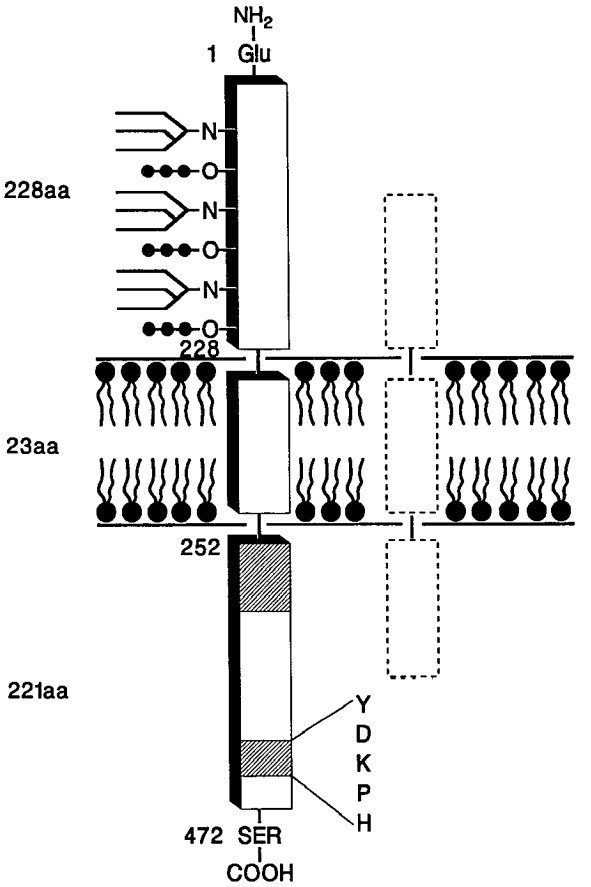


Figure 4 Model of the human IFNγ receptor. The ligand binding chain is a glycoprotein composed of 472 amino acids and is symmetrically oriented around a single 23 amino acid transmembrane domain. The extracellular and intracellular domains are composed of 228 and 221 amino acids, respectively. The protein contains N-linked oligosaccharides and possibly O-linked sugars on some cells. The intracellular domain is rich in serine and threonine residues, some of which are phosphorylated upon interaction of the receptor with ligand in intact cells. Two functionally important regions in the intracellular domain have been identified (shaded area). The membrane proximal region (residues 256–303) is need for both receptor-mediated internalization of ligand and induction of biologic responses. The carboxy terminal region (residues 434–472) is needed only for biologic response induction. The three critical amino acids within the latter region (Y–440, D–441, and H–444) are identified. Functionally active receptors require the presence of a second, as yet undefined, accessory molecule that must be species matched to the IFNγ receptor (shown as the component on the right side of the figure). The suggestion that this component is a membrane protein is based on experiments using human:murine chimeric receptors.

identity to any other known proteins. Specifically, it is neither a member of the Type I cytokine (or hematopoietic) receptor family (characterized by the position of 4 conserved cysteine residues and a membrane proximal WSXWS motif) nor the immunoglobulin superfamily (121). In fact, the sequence identity between the human and murine IFNγ receptors themselves is only 52% (50% identity between the extracellular domains and 55% identity between the intracellular domains). More refined structural analyses of the IFNγ receptor's extracellular domain using predictive algorithms have indicated that the human and murine IFNγ receptors belong to a new family of cytokine receptors (termed the Type II cytokine receptor family) whose members include the receptor for IFNα and tissue factor. The members of this protein family share a similarly organized 210 amino acid binding domain which contains conserved cysteine pairs at both amino and carboxy termini. The Type II receptor family appears to be only distantly related structurally to the Type I family (121)

The intracellular domains of the human and murine IFNγ receptors are 221 and 200 residues, respectively. Both are particularly rich in serine and threonine residues. These two amino acids constitute approximately 25% of all the residues within this domain. Moreover, the intracellular domains of the human and murine proteins contain 6 and 5 tyrosine residues, respectively, five of which are conserved. This observation is particularly noteworthy because of the low overall sequence identity within the human and murine intracellular domains. The receptors' intracellular domains show no significant sequence or structural homologies to any other known receptor polypeptides. Moreover, they do not possess any identifiable kinase, phosphatase, SH2, or SH3 domain characteristics. Therefore, the primary structure analysis of the IFNγ receptor has not provided insights into the mechanism of action of this protein

Functional IFNγ Receptors Require a Species Specific Accessory Component

Expression of the human and murine receptor cDNAs across species boundaries (i.e. in murine and human cells, respectively) confirmed that the cloned cDNAs encoded proteins that bound ligand in the appropriate species-specific manner and internalized it with kinetics that were indistinguishable from natural receptors expressed on homologous cells (107–112). However, the receptors expressed across species lines were unable to induce a functional response in the transfected cells. This result suggested that one or more additional species matched components were required to form a functionally active IFNγ receptor. It also supported the conclusions reached in the seminal experiments of Pestka and colleagues, who in 1987 used murine: human somatic cell hybrids to investigate the minimal

requirements to form a functionally active human IFNγ receptor in murine cells. Fusion of murine and human fibroblasts was known to generate stable cell hybrids that contained the full complement of murine chromosomes but only a random assortment of human chromosomes. All hybrids that contained human chromosome 6, bound human IFNγ. However, responsiveness to the human ligand was observed only in hybrids that contained both human chromosomes 6 and 21. This obligate requirement for two (or more) distinct species-matched gene products led to the hypothesis that functionally active human receptors were composed of at least two distinct polypeptides: the IFNγ receptor itself, responsible for the binding of ligand, and a species matched undefined protein needed for development of functional responses in cells (122). During the past few years, the validity of this hypothesis has been significantly enhanced by the demonstration that expression of the human IFNγ receptor cDNA in murine cells containing only human chromosome 21 leads to formation of a functionally active human IFNγ receptor capable of inducing most if not all IFNγ–dependent biologic responses in the transfected cells (122–124).

Since this accessory factor(s) has not yet been identified, little is known about its structure or function. Using human-murine chimeric IFNγ receptors produced by interchanging the extracellular, transmembrane, and intracellular domains of the human and murine proteins, the site of the species-specific interaction between the receptor and the human accessory protein has been localized to the receptor's extracellular domain (125–127). This result suggests but does not prove that the accessory molecule is expressed at the plasma membrane. In contrast, more is known about the chromosomal location of the gene(s) encoding the human receptor accessory component. Using murine cells containing human chromosome 21 fragments, the accessory component has been localized to a one megabase area on human chromosome 21 in the 21q22 region. Interestingly, this region also contains the gene for the human IFNα receptor (128) (although the IFNα receptor is known not to be the accessory component of the IFNγ receptor). Subsequent experiments utilizing hamster:mouse somatic cell hybrids have indicated that the gene(s) for the murine IFNγ receptor accessory component is located on murine chromosome 16 (129).

Structure-Function Relationships within the IFNγ Receptor

In contrast, considerably more information is available concerning the structure and function of the human and murine IFNγ receptor polypeptides themselves. The ability to reconstitute a functional human IFNγ receptor in murine fibroblasts that contain human chromosome 21 has been used to map the functionally important regions of the human IFNγ

receptor's intracellular domain (123, 130, 133). Full length or truncated (i.e. a receptor lacking all but 3 amino acids of the intracellular domain) receptors were stably expressed in murine fibroblasts containing a single copy of human chromosome 21. The cells expressing the full-length human receptor bound and internalized human IFNγ and responded to it by upregulating expression of MHC class I molecules. In contrast, cells expressing the truncated human receptor bound human IFNγ but neither internalized it nor responded to it. Subsequent experiments that used receptor deletion mutants identified two distinct regions of the intracellular domain which were obligatorily required for receptor function (Figure 4). The first encompassed the 48 amino acids closest to the membrane (termed region I, residues 256–303) and was required for both receptor mediated ligand internalization and degradation and induction of biological responses. The second consisted of the 39 amino acids at the carboxy terminus (termed region IV, residues 434–472) and was required exclusively for biologic response induction (123). Although region I has only been partially characterized to date, a leucine-isoleucine sequence (residues 270–271) has been identified within this region that is involved in effecting receptor-mediated ligand internalization/degradation (130). This sequence matches a motif found in the intracellular domains of the γ and δ chains of the T cell receptor complex and in the cation-dependent mannose–6-phosphate receptor which has been shown to direct the trafficking of these proteins to lysosomes (131, 132). The finding that IFNγ receptor mutants, in which this sequence has been deleted or replaced by alanines, show decreased degradation of bound ligand suggests a similar role for this sequence in directing the IFNγ receptor-ligand complex to a late endosomal or lysosomal compartment

Region IV has been more extensively characterized (133). A point mutational analysis of this region (in which each residue was individually changed to alanine) demonstrated that only three residues are functionally important. These are tyrosine at position 440, aspartic acid at position 441, and histidine at position 444. Alteration of any one of these residues to alanine produced a receptor which was unable to induce a variety of IFNγ-dependent biological responses in murine fibroblasts that contained human chromosome 21. These included induction of IRF–1 (an IFN-inducible transcription factor), MHC class I protein, and nitric oxide. The particular functional importance of tyrosine–440 was confirmed by two additional observations. First, substitution of phenylalanine for tyrosine–440 also resulted in generation of a functionally inactive receptor. This result suggests that the hydroxyl group present on the tyrosine's side chain plays an important role in the signaling process either by (i) forming a structurally critical intramolecular hydrogen bond, (ii) contributing to

intermolecular protein-protein interactions, or (iii) serving as the target for protein tyrosine kinase induced phosphorylation. Second, mutation or deletion of any of the other tyrosine residues within the receptor's intracellular domain did not ablate receptor activity. Additional support for the functional importance of region IV is derived from microinjection experiments in which a monoclonal antibody specific for an overlapping sequence to this region (residues 388–449) inhibited cellular response to IFNγ (134). Microinjection of antibodies that reacted with the middle portions of the intracellular domain (regions II and III) did not inhibit cellular responsiveness. Thus, taken together these studies have identified functionally critical regions within the intracellular domain of the IFNγ receptor. It is expected that the exact roles of these two intracellular domain regions in the signaling/internalization process will soon be elucidated.

Mechanisms of Signal Transduction

Recently, the important observation has been made that IFNγ induces dimerization of its receptor. Using radioligand binding techniques, sucrose density gradient ultracentrifugation, and HPLC gel filtration chromatography, the soluble human IFNγ receptor extracellular domain (ECD) was shown to form a complex with ligand that contained two moles of ECD and only one mole of IFNγ homodimer. The stoichiometry of the complex was dependent on the relative proportions of ligand and receptor added to the reaction mixture. At limiting inputs of IFNγ, a 2:1 (ECD:IFNγ) complex was formed. When ligand was added in vast overabundance, it was possible to demonstrate a 1:1 complex, although even under these conditions the 2:1 complex seemed to be preferred. On the basis of this data, it is likely that the receptor:ligand complex formed physiologically at the cell surface is composed of two receptors bound to one IFNγ homodimer (31, 31a). Support for this concept is derived from recent cross-linking studies, which have shown that under the appropriate conditions, a 2:1 receptor:ligand complex can be immunoprecipitated from human cells treated with physiologic levels of human IFNγ (D. Pennica, D. V. Goeddel, personal communication). A previous report suggested that IFNγ was only capable of producing a 1:1 complex with its receptor (135). However, the recombinant soluble receptor used in those studies was engineered to contain 5 carboxy terminal histidine residues, and it is likely that the presence of these amino acids interfered with the generation of the 2:1 complex (31a).

The physiologic relevance of ligand-induced receptor dimerization is also strongly supported by the finding that functionally inactive receptors act as a dominant negative mutant when overexpressed in homologous cells (32). These studies utilized murine L cells that overexpressed mutant

murine IFNγ receptors lacking either (i) the entire intracellular domain, (ii) the carboxy terminal 39 amino acids (encompassing functional region IV), or (iii) a receptor point mutant in which alanine was substituted for the murine tyrosine residue (residue 420) corresponding to the functionally important human tyrosine-440. Cell lines in which nonfunctional receptors were expressed at levels 100-fold higher than the endogenous receptors no longer responded to murine IFNγ when analyzed in a variety of assays (IRF–1 induction, MHC class I enhancement, nitric oxide induction, and development of antiviral activity). In contrast, comparable overexpression of the wild type murine receptor did not produce a dominant negative effect. Inactivation of IFNγ responsiveness was dependent on the ratio of mutant:endogenous receptors. At 6:1 ratios no inhibition was observed. However, a profound inhibition was seen at ratios of 25:1, and complete inactivation was achieved at a 100:1 ratio. This effect was not due simply to competition for ligand by inactive receptor, because the cells remained unresponsive to ligand even when exposed to IFNγ concentrations 3×10^5 times higher than that normally required to induce a maximal response in wild type cells. Overexpression of inactive receptor did not alter expression of the endogenous receptor. Thus, ligand induced dimerization of the IFNγ receptor, and in particular the formation of a dimeric form of the receptor's intracellular domain may be a critical first step in IFNγ receptor mediated signal transduction.

Subsequent events in the signal transduction pathway are less clearly defined. Whereas some reports indicate that the receptor acts through signal transduction pathways involving protein kinases (136–140), receptor phosphorylation (101, 102), and/or ion fluxes (141), other investigators have suggested that the receptor is primarily responsible for transporting ligand into the cell and propose that intracellular IFNγ somehow induces a cellular response (142–144). Based on the structure-function experiments described above, there is little doubt that receptor-mediated ligand internalization is *not sufficient* to induce biologic responses in cells. This conclusion is based on two observations. First, whereas human IFNγ receptors expressed in normal murine cells effect ligand internalization and degradation with kinetics that are indistinguishable from that of functionally active receptors, no biologic response is observed. Second, mutant human receptors lacking the carboxy terminal 39 amino acids (region IV), when expressed in murine cells containing human chromosome 21, are also functionally inactive despite their ability to mediate ligand internalization. Thus, the molecular explanation for the reports indicating a requirement for ligand internalization remain unclear. It is likely that this uncertainty will soon be resolved when the receptor-related accessory component's identity and function are elucidated and when the mapping of the recep-

tor's intracellular domain region (region I) required for both internalization and function is completed. Finally, the recent exciting identification of key components of the IFNα signaling pathway (145–147) and the observation that the signal transduction pathways utilized by IFNγ and IFNα overlap (148, 149) may provide additional insights that will help define the downstream signalling events and identify the functional branching points that characterize the pleotropic activities of IFNγ.

IFNγ BIOLOGY

Work performed in many laboratories during the past 12 years has unequivocally established that IFNγ is an extremely pleiotropic cytokine that has unarguable physiologic importance in regulating immune and inflammatory processes. To a large extent, this research was made possible by the large scale availability of highly purified recombinant human and murine IFNγ and the generation of neutralizing IFNγ-specific monoclonal antibodies. Most recently, two new and exciting models of genetic IFNγ deficiency in mice have been derived by ablating either IFNγ receptor or IFNγ gene expression using homologous recombination and embryonic stem cell technologies (150, 151). It is expected that these mice will provide new and exciting insights into IFNγ biology.

There is currently a vast amount of information available concerning IFNγ's biologic activities. It is impossible to cover this area in its entirety in this review article. Therefore we have decided to focus on some of the major biologic activities of this cytokine and describe them in the context of three systems which are of prime interest to immunologists: host defense, inflammation, and autoimmunity.

IFNγ'S ROLE IN HOST DEFENSE

IFNγ as a Regulator of Response Induction

Clearly one of the major physiologic roles of IFNγ is its ability to regulate MHC class I and class II protein expression on a variety of immunologically important cell types. These include mononuclear phagocytes, endothelial cells, and epithelial cells, to name a few (57, 62, 152–154). Interestingly, although IFNγ acts to increase class I and class II expression on most cells, it inhibits class II expression on B cells (155). Whereas IFNα and IFNβ can also upregulate class I expression on cells, they are not inducers of MHC class II proteins. IFNγ's ability to perform these functions was first appreciated in in vitro experiments in which purified recombinant IFNγ was added to primary cells or cultured cell lines and MHC protein expression monitored using immunochemical techniques (152).

Subsequently, this observation was confirmed using two types of in vivo approaches. In the first, IFNγ was injected directly into the host and MHC antigen expression monitored. These experiments showed that IFNγ could upregulate MHC class I and class II proteins both locally at the injection site and systemically. In the second type of experiment, the host was injected with IFNγ-specific, neutralizing monoclonal antibodies and then exposed to an immunologic challenge such as a parasitic infection. Whereas the tissues and cells of control animals displayed significant induction of MHC class II antigens on their surface, cells from anti-IFNγ treated animals did not. The latter experiment was particularly informative because it documented that endogenously produced IFNγ plays an important physiologic role in regulating MHC protein expression (81, 156). More detailed analyses have shown that IFNγ *enhances* MHC class I expression (2–4 fold) on cells that constitutively express class I and *induces* class II expression on cells that are normally devoid of these proteins (157). At the molecular level, IFNγ has been shown to exert its activity by regulating MHC gene transcription. MHC class I and class II genes contain *cis*-acting elements in their promoter regions that bind to IFNγ-induced *trans*-acting factors. The molecular nature of these elements and factors are currently being elucidated (158, 159). At the functional level, IFNγ-dependent upregulation of MHC gene expression is an important step in promoting antigen presentation during the inductive phase of immune responses (160–164). On most cells, IFNγ is sufficient to upregulate MHC molecules. However, its action is often synergistically enhanced by additional endogenous stimuli such as TNF or exogenous agents such as bacterial or microbial products. Importantly, some cells such as pancreatic islets display an obligatory requirement for the combined actions of IFNγ and TNF (165–167).

The monocyte/macrophage is a prime cellular target for IFNγ under physiologic conditions. Work from several laboratories has indicated that IFNγ is one of the major cytokines responsible for activating or otherwise regulating the differentiation and function of mononuclear phagocytes (58, 168). IFNγ has been shown to effect the differentiation of immature myeloid precursors into mature monocytes. It promotes antigen presenting activity in macrophages, not only through the induction of MHC class II expression, but also by increasing levels of several intracellular enzymes that may be important for antigen processing (169, 170). In addition, IFNγ augments expression of macrophage cell surface proteins such as ICAM–1 that enhance the functional consequences of the interaction between macrophages and T cells during antigen presentation (171–173)

IFNγ also exerts its effects on other cells of the immune system. It regulates immunoglobulin isotype switching on B cells (174) and anta-

gonizes the ability of IL–4 to induce MHC class II expression on murine B cells (175). These responses result from the direct effect of IFNγ on the B cell. B cell responses are also influenced indirectly by IFNγ's ability to regulate the development of specific subsets of $CD4^+$ T cells. IFNγ has a profound antiproliferative effect on the T_H2 subset of murine $CD4^+$ T cells but not on T_H1 (61, 63). The ability to regulate $CD4^+$ T cell activation/differentiation thereby establishes IFNγ as a key component in determining the type of immune effector function that eventually develops during the course of an immune response (Figure 5). The opposing effects of IL–10 and IFNγ thereby serve to cross-regulate the development of specific immune responses. IL–10 inhibits IFNγ production by T cells and NK cells and thereby diverts the response to the humoral pole. In contrast, IFNγ inhibits the expansion of T_H2-like T cells, thereby eliminating a key cellular source of IL–10. This event then diverts the response to the cell-mediated immunity pole. The suggestion has been made that IFNγ serves a positive role in the generation of $CD8^+$ cytolytic T cells (CTL) (176). However, this hypothesis has not yet been stringently demonstrated and, in fact, mice with genetic IFNγ-unresponsiveness produce more CTL activity during a mixed leukocyte reaction than their normal counterparts (150, 151). This result indicates that IFNγ may actually exert an antiproliferative

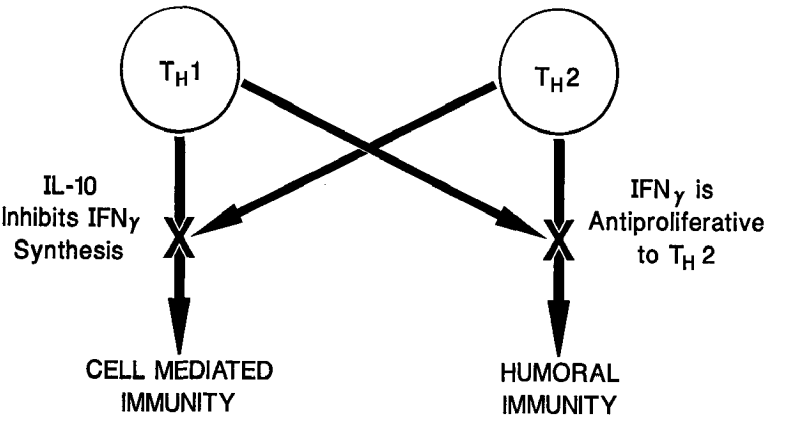


Figure 5 IFNγ and IL–10 cross-regulate the development of specific arms of immune effector functions. IL–10 inhibits IFNγ production by TH1 $CD4^+$ T cells and NK cells and thereby inhibits development of cell-mediated immune responses. At the same time IL–10's actions promote development of humoral immunity. In contrast, IFNγ inhibits the proliferation of TH2 $CD4^+$ T cells thereby inhibiting production of TH2 derived cytokines including IL–10. This regulatory action results in the preferential development of cell mediated immunity and the depression of humoral immunity. The identity of the signals that lead to the predominance of IL–10 versus IFNγ effects remain undefined.

effect on the $CD8^+$ T cell population, as well as the $CD4^+$ T_H2 cell population

IFNγ as a Regulator of Effector Mechanisms

There can be little doubt that IFNγ is the major physiologic macrophage activating factor (MAF) and therefore is the primary cytokine responsible for inducing nonspecific cell mediated mechanisms of host defense. Work from several laboratories has unequivocally established IFNγ's ability to activate nonspecific cytocidal activity in macrophages toward a variety of intracellular and extracellular parasites and neoplastic cells (54, 80, 177). IFNγ (i) induces the expression of, as yet undefined, structures on macrophages that recognize target cells and (ii) promotes the elaboration of macrophage-derived cytocidal compounds such as reactive oxygen– and reactive nitrogen–intermediates and TNFα (178). IFNγ also reduces the susceptibility of macrophage populations to microbial infection (81, 179). The importance of IFNγ in the clearance of microbial pathogens has been amply demonstrated using animal models. Mice pretreated with neutralizing monoclonal antibodies to IFNγ lose their capacity to resolve infection initiated with a sublethal dose of a variety of microbial pathogens such as *Listeria monocytogenes* (80, 81, 156), *Toxoplasma gondii* (180) or *Leishmania major* (181). These experiments thus document the capacity of endogenously produced IFNγ to activate macrophages under physiologic in vivo conditions

Currently a great deal of attention is being focused on IFNγ's ability to induce nitric oxide (NO) production in cells. NO is a cellular product that appears to play an important role in effecting intracellular killing of microbial pathogens in the mouse. Nitric oxide is generated as a result of the enzymatic conversion of L-arginine to L-citrulline (182). This reaction is catalyzed by a family of enzymes known as nitric oxide synthase (NOS). At least 3 forms of the enzyme have been identified (183). Two are expressed constitutively in a tissue-specific manner (endothelium and nervous tissue) and produce low levels of NO that function to effect cell-cell communication. In contrast, the third is an inducible form of the enzyme whose expression is controlled by two stimuli: IFNγ and a second signal. The signals that trigger NO production in IFNγ primed cells are a diverse group of endogenous and exogenous substances such as TNFα, IL–1, LPS, and whole bacteria (184, 185). The array of components that trigger NO production in cells varies depending upon the nature of the NOS producing cell. For example, in macrophages NOS is induced by IFNγ and TNF but not IL–1, while in certain fibroblasts or pancreatic islets NOS is induced by IFNγ and either TNF or IL–1 (185, K. C. F. Sheehan, R. D. Schreiber,

unpublished observations). The inducible macrophage NOS has recently been cloned and characterized (186–188). Whether the inducible NO synthase expressed in other cell types is identical to the macrophage enzyme needs to be established.

IFNγ-dependent formation of nitric oxide appears to be a major mechanism in the mouse for the macrophage-mediated killing of intracellular pathogens. Much of this information has been gleaned from the study of murine models of infection with *Leishmania* or *Listeria*. Macrophages exposed in vitro to IFNγ and infected with either *Leishmania* amastigotes or *Listeria* develop the capacity to kill the intracellular pathogens. Killing is completely inhibited when the macrophages are treated with competitive inhibitors of NOS such as the L-arginine analogs, N-monomethyl-L-arginine (L-NMMA) (181) or aminoguanidine (189). In vivo, the important role of IFNγ-dependent induction of NO in murine models of microbial immunity has been indicated by at least three types of experiments. First, mice undergoing active infection produce NO as detected by the presence in the urine of the stable (NO) oxidation product nitrite (NO_2). NO_2 production was blocked when the mice were treated with neutralizing monoclonal antibodies specific for either IFNγ or TNF (181). Second, killing of live *Leishmania* in vivo at a local site (footpad) was significantly decreased when the animals were treated with the NOS inhibitor NMMA (190). Third, and perhaps most significant, is the recent observation that mice treated with aminoguanidine succumb to infection with a sublethal dose of *Listeria monocytogenes*, much like mice treated with anti-IFNγ (K. Beckerman, H. Rogers, C. Tripp, J. Corbett, R. Schreiber, M. McDaniel, E. Unanue, manuscript in preparation). Interestingly, human mononuclear phagocytes have not been shown to produce nitric oxide or NOS thus far (although human islets and hepatocytes can indeed produce NO). Whereas this latter observation may indicate that species specific differences exist in IFNγ-dependent macrophage mediated cytocidal responses, the possibility must also be considered that the human mononuclear phagocyte requires an additional signal to induce NOS.

In addition to enhancing *nonspecific* cell mediated cytocidal activities, IFNγ also enhances the ability of the macrophage to participate in other immune response effector functions. It increases expression of high affinity Fc receptors on monocytes/macrophages (FcγRI) and thereby enhances the capacity of these cells to participate in antibody dependent cellular cytotoxicity (ADCC) reactions (191). IFNγ also enhances the biosynthesis of a variety of complement proteins (such as C2, C4, and Factor B) by macrophages and fibroblasts (192) and regulates the expression of complement receptors on the mononuclear phagocyte plasma membrane

thereby promoting humoral immunity through enhancement of complement activity.

IFNγ IN THE INFLAMMATORY RESPONSE

A substantial amount of data is now available that supports the concept that cytokines play a major role in promoting inflammatory responses. IFNγ also participates in this process largely through its ability to enhance TNF production and/or activity. The focus of this section is therefore to highlight the important synergistic action of IFNγ and TNFα in inflammation.

IFNγ Regulation of TNFα Production

It is now well established that LPS stimulated macrophages produce increased amounts of TNFα when concomitantly treated with IFNγ (193–196). A few early studies indicated that IFNγ could directly induce TNFα production in macrophages. However, this result was not substantiated in other laboratories, and it is likely that the induction was a result of increased sensitivity of IFNγ-treated cells to low levels of LPS present in the medium. IFNγ can cause the upregulation of LPS-induced TNFα production in a variety of different murine macrophage populations, including resident and elicited peritoneal exudate macrophages and bone marrow derived macrophages grown in vitro. Thus the ability of IFNγ to enhance LPS-induced TNFα production is not strongly dependent on the activation state of the macrophage. Thioglycollate-elicited macrophages concomitantly stimulated with a mixture of LPS and IFNγ displayed 6–8 times higher steady-state levels of TNFα specific mRNA than cells treated with LPS alone. Maximal levels of TNFα mRNA were achieved 2–4 hours after exposure to either LPS alone or to the LPS/IFNγ mixture (193, 194). IFNγ also enhanced the ability of LPS to induce the TNFα protein (as measured 16 hr after stimulation). Enhancement of TNFα production was most evident in cells pretreated for 4 hr with IFNγ but was still detectable even when IFNγ was added 6 hr after LPS stimulation (193). IFNγ can also correct the genetic defect in macrophages derived from the LPS unresponsive C3H/HeJ mouse. C3H/HeJ macrophages do not produce TNFα when stimulated either in vivo or in vitro with moderate concentrations (1 μg/ml) of LPS. However, thioglycollate-elicited macrophages from these mice treated with physiologic concentrations of purified recombinant murine IFNγ, produce both TNFα mRNA and protein when stimulated with LPS at 1 μg/ml (193).

Several studies indicate that the enhancement of LPS-induced TNFα production by IFNγ is a result, at least in part, of increased transcription

of the TNFα gene (193–195, 197, 198). However, the suggestion has also been made that, in this system, IFNγ may also increase TNFα mRNA stability. In part, this latter hypothesis is based on the demonstration that TNFα mRNA contains an octomeric sequence (UUAUUUAU) within its 3′ untranslated region that is a point of attack for a selective nucleolytic activity capable of hydrolyzing mRNA. Similar sequences have also been found in the 3′ untranslated regions of other short-lived cytokine mRNAs such as those that encode IL–1 and GM-CSF. Independent studies have shown that the half-life of TNFα mRNA is increased in cycloheximide-treated cells stimulated with LPS. Therefore it seems likely that IFNγ may exert some of its enhancing effects on TNFα production by inhibiting the generation of this short-lived repressor activity (199).

IFNγ has also been shown to enhance expression of TNFα receptors on a variety of different cell types (200–202). Treatment of cells with IFNγ does not alter their ligand binding affinity but increases receptor expression 3–5 fold. Both types of TNFα receptors (p55 and p75) can be increased by IFNγ. Enhanced receptor expression appears to be due to increased protein synthesis and not to translocation of receptors from an intracellular pool since IFNγ's effects can be blocked by treatment of the cells with actinomycin D. However, the biologic significance of this effect remains to be established, because no correlation has yet been found between the level of TNFα receptor expression and the magnitude of the cellular response induced (200, 201, 203)

Cellular Recruitment

During an inflammatory response, cells leave the circulation and migrate to the point of infection. During this process they must first bind to and then extravasate through the vascular endothelium. IFNγ and TNFα can promote the expression of overlapping sets of cell-surface molecules that play an important role in this process (171). Studies using cultured human umbilical vein endothelial cells (HUVEC) demonstrated that in vitro treatment with IFNγ induces a significant but moderate increase in ICAM–1 (5-fold) and MHC class I molecules (11-fold) but no induction of ELAM–1 (171, 204). In the same setting, TNFα provoked a dramatic enhancement of ICAM–1 expression (40-fold) as well as moderate increases in ELAM–1 and MHC class I proteins. However, when HUVEC were cultured in the presence of both IFNγ and TNFα, the expression of all three molecules was enhanced in a synergistic manner with ICAM–1 and class I showing 60- and 24-fold increases, respectively. In addition, the combination of IFNγ and TNFα induced ELAM–1 expression on a significantly higher percentage of cells than TNFα alone. In the presence of both cytokines ELAM–1 also displayed a longer half-life at the cell surface. This latter

effect may result in a prolongation of the endothelial cell's ability to recruit circulating lymphocytes (204). Thus, the ability of IFNγ and TNFα to enhance expression of cell surface adhesion molecules may serve to expand and amplify the overall inflammatory response.

The cooperative ability of IFNγ and TNFα to modulate cell migration was confirmed in in vivo experiments (205). Skin biopsies, taken from baboons treated intracutaneously with both IFNγ and TNFα were found to contain twice the numbers of monocytes compared to animals injected with either cytokine alone. Expression of ELAM–1, ICAM–1, and MHC class I molecules was also synergistically enhanced in animals treated with both cytokines.

Shock

TNFα has long been known to mediate many of the toxic effects of LPS (206–208) and is a key mediator in the Shwartzman reaction (a model of LPS-mediated tissue damage) (209, 210). In the classical Shwartzman reaction, animals are initially treated with a local, sensitizing dose of LPS (5 μg), followed 24 hr later with a provocative intravenous dose of LPS (100 μg). The physiologic responses evoked by this protocol mimic those seen clinically in septic shock and disseminated intravascular coagulation and include hemorrhagic necrosis, fibrin- and platelet mediated vascular occlusion and accumulation of neutrophils at the local site (206, 207, 211). Using the Shwartzman reaction as a model to investigate the mechanism(s) involved in LPS-induced disease, several investigators have shown that IFNγ plays a crucial role in the progression of this inflammatory response (209, 212–217). Treatment of animals with IFNγ prior to sensitization with LPS leads to enhanced production of TNFα and increased mortality (212, 213, 218). Conversely, treatment of animals with neutralizing IFNγ-specific monoclonal antibodies prior to injection of the sensitizing dose of LPS, protects them from the pathologic effects of the provocative dose (215). It is possible that the IFNγ produced in this reaction is derived from NK cells in a manner resembling that described above for the SCID mouse. The sensitizing dose of LPS may stimulate resting macrophages to produce TNFα. TNFα and LPS then may stimulate NK cells to produce and secrete IFNγ. NK cell–derived IFNγ then should prime other macrophages in the vicinity to produce copious amounts of TNFα (and other inflammatory mediators such as IL–1 and IL–8) when exposed to the provocative dose of LPS. Once produced, the large amounts of TNFα then would induce a cascade of reactions that have immunopathologic consequences. Thus, in this model, IFNγ serves a crucial intermediary role in activation of effector macrophage populations. A similar role for IFNγ was noted using a model of endotoxin shock (218). Mice pretreated with recombinant murine IFNγ

18 hr prior to intravenous injection with an LD_{50} dose of LPS showed significantly increased mortality (92% dead at 72 hours). Conversely, mice treated with neutralizing IFNγ-specific antibodies prior to administration of an LD_{90} dose of LPS were protected (218) from LPS induced lethal shock. Thus, LPS-induced IFNγ appears to be a key mediator in the development of the immunopathologic consequences of inflammatory responses.

IFNγ IN AUTOIMMUNITY

Although IFNγ appears to play a participatory role in the development of some autoimmune processes, different experimental models have yielded conflicting interpretations as to its immunopathologic versus protective roles. One model in which IFNγ has been implicated as a causative agent is the development of autoimmune nephritis in the (NZB × NZW)F1 mouse, a syndrome which produces a pathology similar to that seen in the human disease, systemic lupus erythematosis. Administration of exogenous IFNγ to (NZB × NZW)F1 mice accelerated the progression of spontaneous glomerulonephritis (50% survival = 9.5 months for control animals and 7.5 months for IFNγ treated mice) (219–221). Conversely, animals treated with IFNγ-specific monoclonal antibodies displayed significant remission and increased survival (220). Additional support for IFNγ's role in producing tissue specific damage that leads to autoimmune responses has been obtained from experiments utilizing transgenic mice expressing the IFNγ gene under the control of the rat insulin promoter (222). These mice develop a severe insulitis and become hypoglycemic and thereby develop a syndrome that appears very much like insulin dependent diabetes mellitus, which is commonly thought to be an autoimmune disease.

In contrast, IFNγ plays a protective role in certain murine models of experimental autoimmune encephalomyelitis (EAE). This demyelinating disease can be generated either by active immunization with myelin basic protein (MBP) in adjuvant or by the adoptive transfer of encephalogenic MBP-specific $CD4^+$ T cell clones (223–227). Immunization of C57BL/6 or SJL/J mice with MBP results in moderate or lethal forms of the disease, respectively (227). Treatment of C57BL/6 (moderate disease) mice with anti-IFNγ exacerbates disease and leads to increased mortality. Conversely, treatment of SJL/J mice (which normally exhibit 100% mortality) with IFNγ led to enhanced survival. Thus, using the active immunization protocol, IFNγ appears to downregulate the development of EAE. This protective role is not observed when EAE is induced using the passive T cell transfer protocol. Moreover, administration of IFNγ to human

subjects with active multiple sclerosis (the natural human disease that resembles murine EAE) leads to an exacerbation of the disease (228). Together, these results demonstrate that IFNγ can play different roles in the development of various autoimmune responses and may act through a variety of different mechanisms. Further experimentation is required to determine the molecular mechanism(s) responsible for the seemingly contradictory roles of IFNγ in autoimmune disease.

CONCLUDING REMARKS

In this chapter we have reviewed some of the recent developments that have significantly added to our understanding of the biochemistry and biology of IFNγ. To a large extent, this progress was made possible by the technological advances that have occurred within recent years in the fields of molecular genetics, protein chemistry, and cell biology. We now know a great deal about the structure of the IFNγ molecule and its receptor, and we have begun to identify which of the functional activities of the protein are physiologically relevant. Nevertheless the molecular mechanisms that underlie IFNγ's pleotropic activity remain undefined, and we still have not yet found a means to fully develop the protein's therapeutic potential. It is likely that these subjects will be the focus of future IFNγ research.

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THE Ly–49 AND NKR-P1 GENE FAMILIES ENCODING LECTIN-LIKE RECEPTORS ON NATURAL KILLER CELLS: The NK Gene Complex*

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Abstract

Natural killer cells lyse tumor and virally infected cells in a specific manner that has not been molecularly characterized. Target cell expression of major histocompatibility complex (MHC) class I molecules is correlated with target cell resistance to natural killing. A mechanism to explain this observation is that NK cells may display two types of recognition and activation molecules that have opposing functions when bound to target cell ligands. One type of surface receptor such as the NKR-P1 molecule may activate NK activity whereas the other, represented by the mouse Ly–49 molecule, may engage target cell MHC molecules and inhibit cytotoxicity by transducing "negative" signals. NKR-P1 and Ly–49 are structurally related, and they are encoded by genetically linked loci in a chromosomal region, termed the NK gene complex (NKC), on distal mouse

chromosome 6. Target cell susceptibility to natural killing may be dependent upon specific ligand-receptor interaction with these activating or inhibitory NKC-encoded molecules.

INTRODUCTION

Natural killer (NK) cells constitute a small population of lymphocytes that typically display a large, granular morphology. They are found in blood and in peripheral lymphoid tissues, particularly the spleen. NK cells were initially defined functionally, by their ability to kill certain tumor and virally infected cells but not normal cells (1–3). They may thus participate in host defense against malignancy and viral invasion. NK cells are also capable of rejecting allogeneic or parental bone marrow stem cells and may therefore be important in marrow transplantation.

When it became possible to identify B and T lymphocytes on the basis of surface markers, NK cells were found to differ from either of these lymphocyte subsets (4). Moreover, NK cells lack B and T cell antigen receptors, i.e. surface immunoglobulin, the hallmark of B cells, and the T cell receptor (TCR). On T cells, the TCR is expressed in association with the molecular complex, CD3 (5). Although freshly isolated NK cells express the ζ chain of the CD3 complex (6, 7), they do not display other components of the complex, do not express mRNA for mature TCR chains, and do not rearrange TCR genes (4, 6, 7).

These observations indicate that NK cells must recognize target structures through receptors that differ from the TCR. This conclusion is supported by marked functional differences between T cells and NK cells in their requirement for major histocompatibility (MHC) antigens on target cells. Whereas T cells recognize antigenic peptides only when they are bound to MHC antigens (8), NK cells effectively lyse targets that lack MHC class I antigens (9, 10). Indeed, natural killing is often inhibited by the expression of MHC class I antigens on targets. Because physical contact between NK cells and targets is required for killing, the molecular basis for killing and for target specificity very likely involves specific interactions between cell surface molecules expressed by effector and target cells. Although target recognition must be possible in the absence of MHC antigens, the inhibitory effect of MHC antigens on killing must be explained; the expression of MHC antigens on target cells must either disrupt the recognition of other target structures or it must initiate inhibitory signals in NK cells (9).

Recently, two families of receptor-like surface molecules have been identified on NK cells. These families are genetically linked and share structural features, but they appear to have opposing effects on NK cell

activity. The members of one family activate natural killing. They include NKR-P1 in the rat (11), and NK1.1 in the mouse (12). MAbs directed against these structures activate NK cell lysis (11, 13), and they initiate biochemical events (14) that are reminiscent of NK activation in natural killing (14, 15). These findings suggest that members of the NKR-P1 gene family may serve as receptors on NK cells, but the molecular structures that are recognized by NKR-P1 have not been identified. The other receptor family is represented by mouse Ly–49 (16). In contrast to NKR-P1, Ly–49 delivers inhibitory signals to NK cells (17). Our recent studies have demonstrated that the apparent engagement of Ly–49 on the NK cell by specific MHC class I antigens on the target cell inactivates NK cells (17).

The NKR-P1 and the Ly–49 gene families thus appear to encode receptors on the NK cell surface that deliver opposing NK cell activation signals. Both gene families lie in close proximity on the mouse 6th chromosome (18), an area that we have called the NK gene complex (NKC). In this monograph, we review the genetics, structure, and function of these receptor-like molecules encoded in the NKC.

LY–49

Structure and Expression

Ly–49 is a dimeric glycoprotein expressed on a subpopulation of NK cells in certain strains of mice, including C57BL/6 (16). Although Ly–49 is not expressed on fresh T cells, it was originally demonstrated on certain T cell tumors by the monoclonal antibody (mAb) A1 (19). We (20) and others (21) cloned a full length (1205 bp) cDNA for Ly–49. In transfected eukaryotic cells, the cDNA directs the expression of an 85-kDa disulfide-linked homodimer with 44-kDa subunits (20). The expressed homodimer reacts with mAb A1 and with two rat monoclonal antibodies, YE1/48, and YE1/32. The derived amino acid sequence reveals that the putative Ly–49 polypeptide has features of a type II integral membrane protein (extracellular carboxy terminus) (20, 21).

Most of the extracellular domain of Ly–49 demonstrates homology to the C-type lectin supergene family (20). Members of this family include the asialoglycoprotein receptor, the low-avidity IgE receptor (CD23), and the selectins (MEL14/LAM–1, ELAM–1, and CD62) (22–24). While members of the C-type lectin supergene family are only weakly homologous to each other, they share structural features, including conservation of six extracellular cysteines. The deduced sequence of Ly–49 demonstrates the structural features of these receptors, but it is not strongly homologous to any of them; Ly–49 is $\sim$23% homologous to the asialoglycoprotein recep-

tor (20) and to murine CD23 (25). Thus, Ly–49 appears to be a novel member of the C-type lectin supergene family.

The known ligands for almost all members of the C-type lectin supergene family are carbohydrates, and ligand binding is calcium-dependent. The single exception is CD23, which binds IgE in a manner that is independent of carbohydrate expression on IgE (26). The avidity of CD23 for IgE is low ($k_a \leqslant 10^{-8}M$), and recent findings indicate that CD23 recognizes additional ligands in a calcium-dependent manner, namely carbohydrate moieties on CD21 (27, 28). Thus, the homology of Ly–49 to C-type lectins suggests that the ligand for Ly–49 is a carbohydrate, but it is also possible that Ly–49, like CD23, can bind noncarbohydrate structures. Regardless of its ligand, the homology of Ly–49 to C-type lectins suggests that Ly–49 may be a cell surface receptor. The limited expression of Ly–49 on a subset of NK cells suggests further that Ly–49 may play a particular role in the function of NK cells.

Ly–49 Is Encoded by a Member of a Multigene Family

Southern blot analysis with a 200-bp Ly–49 cDNA probe demonstrates multiple restriction fragments of genomic DNA (20). We (20) and others (21) have therefore hypothesized that Ly–49 is encoded by a member of a family of highly related genes. In our ongoing studies to explore this possibility, we have used Ly–49 cDNA to isolate cross-hybridizing transcripts from a cDNA library prepared with mRNA from unfractionated, C57BL/6J-derived, IL–2–activated NK cells. Early results from these studies have confirmed the presence of at least three transcripts that are closely related to, but distinct from, Ly–49 (H. S. Smith, W. M. Yokoyama, unpublished observations). As an example, the nucleotide sequence of one clone (clone 5A) is 87% identical to that of Ly–49, while the Ly–49 and clone 5A proteins are identical at 76% of amino acid residues. (5A cannot represent an allelic polymorphism of Ly–49 because both cDNAs were obtained from C57BL/6–derived libraries.) Ly–49 is thus much more closely related to 5A than it is to other members of the lectin supergene family, which usually have amino acid identity at $<35\%$ of residues and little or no significant nucleotide identity. Because of their close homology, we consider that the Ly–49 and 5A cDNAs represent transcripts from members of the same gene family. In addition, we have found cDNAs that appear to represent deletional variants of each other (H. S. Smith, W. M. Yokoyama, unpublished observations). These deletions may be alternatively spliced transcripts, but this has not been confirmed.

Additional forms of the Ly–49 molecule have been described in a cDNA library derived from a (C57BL/6 × CBA)F_1 hybrid mouse (29). The cDNA clones revealed at least three additional forms. However, the parental

strains display distinct RFLP variants for Ly–49 (16). This raises the possibility that some of these forms of Ly–49 may represent allelic variants rather than distinct isoforms in the same individual (strain). Further analysis is required to characterize the extent of polymorphism in the Ly–49 gene family.

Function of Ly–49

THE EXPRESSION OF CLASS I MHC ANTIGEN ON TARGET CELLS INHIBITS NATURAL KILLING The molecules on the surface of target cells that are recognized by NK cells have not been identified. Results of most early studies led to the general concept that the lytic activity of heterogeneous populations of NK cells is not restricted by expression of specific MHC alloantigens on target cells (1–3). However, more recent studies suggest that expression by target cells of certain MHC class I molecules correlates inversely with susceptibility to NK cell-mediated lysis.

Several groups have generated MHC class I–negative cell lines and have found such mutants to be more susceptible to NK cell-mediated lysis than the parental cells (30–32). Moreover, reconstitution of MHC class I expression, by transfection of the β_2-microglobulin gene, restores resistance to NK cell–mediated lysis (33). Although not all investigators have found such a correlation (34, 35), recent studies involving gene transfer in human cells (32, 36) suggest that expression of only certain MHC class I molecules is related to the resistant phenotype.

These findings in vitro are corroborated by in vivo experiments using β_2-microglobulin-deficient transgenic mice. Cells from these mice, which lack expression of class I MHC antigens, are notably susceptible to NK cell–mediated lysis, and bone marrow from these mice cannot engraft lethally irradiated, MHC-matched mice unless the recipients are depleted of NK cells (37–39).

This role for NK cells in the rejection of bone marrow stem cells resembles the phenomenon of hybrid resistance. In hybrid resistance, inbred mouse parental bone marrow is rejected by lethally irradiated F_1 hybrid recipients through a mechanism that requires NK cells (40). Through the use of congenic resistant mice as donors, it has been possible to map genes that, when expressed in donor bone marrow cells, result in their rejection. The primary locus, *Hh–1*, has been mapped to the MHC region, near the H–2D locus (41). Hybrid resistance is recessive; both alleles must confer resistance or the marrow engrafts the host. An alternative explanation is that the products of *Hh–1* actively prevent rejection by inhibiting natural killing, and this effect is dominant.

How might the effect of class I MHC antigens on natural killing relate to hybrid resistance? Perhaps *Hh–1* encodes MHC-like surface antigens

that inhibit natural killing. Mapping studies (41) have placed *Hh–1* centromeric to H–2D, but other evidence indicates that H–2D itself can influence hybrid resistance. Thus, (H–2D^b × H–2D^d)F$_1$ hybrid mice normally reject bone marrow cells from H–2^b mice, but they accept bone marrow cells from H–2^b mice that are transgenic for H–2D^d (42). This finding is compatible with the possibility that the MHC class I antigen H–2D^d inhibits hybrid resistance. Further studies are needed, however, to examine the relation between hybrid resistance and the inhibitory effect of MHC class I antigens on natural killing.

MHC class I antigens may constitutively bind allele-specific endogenous peptides in the peptide-binding groove formed by the α1 and α2 helices (43, 44). It is possible, therefore, that a specific peptide, binding to the appropriate MHC antigen, is a key determinant in inhibiting NK cell lysis. In support of this possibility, MHC class I exon shuffle experiments and mutational analysis have implicated the peptide-binding cleft in this inhibition (32, 36, 45). Thus, specific MHC-bound peptides on target cells, as well as the class I MHC antigens themselves, may significantly influence NK cell function.

As noted above, several mechanisms have been proposed to account for the inverse correlation of target cell MHC antigen expression and susceptibility to NK cell lysis (9, 10). The MHC antigen may "mask" the target cell antigen responsible for triggering NK cells, thus blocking recognition by NK cells. Alternatively, the MHC antigen may be specifically recognized by an effector cell molecule, triggering a "negative" signal. Our recent studies support the latter possibility (17).

LY–49$^+$ NK CELLS ARE INHIBITED BY MHC CLASS I ANTIGENS H–2D^d Because Ly–49 is expressed by a distinct subpopulation of NK cells (16) and is encoded by a member of a multigene family that displays allelic polymorphism (20), we suspected that Ly–49 itself may be involved in NK cell activity as an immune recognition structure. We therefore studied whether the specificity of Ly–49$^+$ NK cells differed from Ly–49$^-$ NK cells (17). These two subpopulations were obtained from IL–2–activated (800 U/ml), T cell–depleted, C57BL/6-derived NK cells; Ly–49$^+$ cells were isolated by panning with mAb A1, while Ly–49$^-$ cells were obtained by complement-mediated lysis using the same mAb. Both subpopulations of cells were incubated overnight in IL–2 (800 U/ml), washed, and then, to minimize any effect of the isolation procedure itself, cultured for an additional 3 days. The isolated Ly–49$^+$ and Ly–49$^-$ IL–2–activated NK cells were uniformly positive or negative, respectively, for Ly–49 expression as determined by FACS analysis. Otherwise, these subpopulations had an identical phenotype with respect to reactivity with Abs specific for NK1.1, FcγRIII,

and asialoGM$_1$. The subpopulations also demonstrated similar levels of cytotoxicity against the NK-sensitive tumor YAC–1, and both subpopulations were capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) (17). Thus, we succeeded in isolating subpopulations of IL–2–activated NK cells that differ in most regards only in their expression of Ly–49.

Further examination of these NK cell subpopulations demonstrated differences in their ability to kill certain tumor cells (17). In particular, several murine tumor targets are effectively lysed by Ly–49$^-$ NK cells, but not by Ly–49$^+$ NK cells (Table 1). The reciprocal has not been found; no tumors are lysed by Ly–49$^+$ NK cells but not Ly–49$^-$ NK cells. Some tumors are resistant to lysis by both Ly–49$^+$ and Ly–49$^-$ cells. In the case

Table 1 Target cell specificity of Ly-49$^+$ IL-2-activated NK cells

Cell line	Type	H-2	Specific lysis by Ly-49$^+$ NK cells[a]	Specific lysis by Ly-49$^-$ NK cells
YAC-1	T cell	a	+++[b]	+++
IC-21	macrophage	b	++	++
B16S	melanoma	b	+++	+++
C1498	lymphoma	b	++	++
WEHI-3	macrophage	d	–	–
BB88	leukemia	d	–	–
J774	macrophage	d	–	+++
P388D1	macrophage	d	–	+++
RAW264.7	macrophage	d	–	+++
PU5-1R	macrophage	d	–	+++
WEHI 265.1	macrophage	d	–	+++
WR19M.1	macrophage	d	+/–	++
RAW309Cr.1	macrophage	d/b	–	+
SP2/0	B cell	d	–	+++
LB27.4	B cell	d/b	–	+
L5178Y-R	leukemia	d	–	+++
T27A	leukemia	d	–	+
D2N	leukemia	d	–	++
DAP3	fibroblast	k	–	++
BW5147.G.1.4	T cell	k	–	++
R1.1	T cell	k	–	+
RIE/TL8x.1	T cell	k[c]	++	++

[a] C57BL/6J, IL-2-activated NK cells were separated according to Ly-49 expression with mAb A1 (anti-Ly-49) as described in text.

[b] Specific lysis by Ly-49$^+$ or Ly-49$^-$ IL-2-activated NK cells was determined at varying effector:target ratios in standard, 4 h ^{51}Cr-release assay. Results shown are specific cytotoxicity at the 25:1 E:T ratio (0–10% = – or +/–, 10–25% = +, 26–50% = ++, 51–75% = +++, 76–100% = ++++).

[c] Derived from R1.1 and lacks MHC class I expression because of defect in β_2-microglobulin.

of the human tumor cell Daudi, which expresses Fc receptors, lysis by both NK subpopulations can be induced by redirected lysis with mAb that bind the Fc receptors on Daudi (through their Fc portion) and that recognize activation antigens on NK cells, including NK1.1, Ly–6, or VEA antigens (13). For the murine tumors that are selectively resistant to lysis by Ly–49$^+$ NK cells, however, cytotoxicity cannot not be induced by any of these antibodies (17), even when the resistant targets express Fc receptors (e.g., J774, P388D1). Moreover, Ly–49$^+$ NK cells are unable to lyse these resistant targets under conditions in which ADCC or lectin (Con A)-mediated cytotoxicity can be demonstrated using the Ly–49$^-$ effector population (17, 46). Thus, expression of Ly–49 by NK cells appears to correlate with the inability of NK cells to kill certain tumor targets by any of a variety of mechanisms. These findings suggest that Ly–49 inactivates cytotoxicity by NK cells against specific targets.

To further understand the mechanism by which Ly–49 regulates NK cell activity, we attempted to identify a ligand for Ly–49. Because Ly–49 belongs to a family of closely related molecules that displays allelic polymorphism, we suspected that the ligand for Ly–49 might display similar complexity. Tumor targets that are specifically resistant to lysis by Ly–49$^+$ NK cells represent several different cell lineages, including B cells, T cells, macrophages, and fibroblasts. Thus, the apparent specificity of Ly–49$^+$ NK cells is not related to target cell type. A survey of the H–2 haplotypes of the informative tumor targets (Table 1) reveals a striking correlation between the H–2^k and H–2^d expression by the target cells and their resistance to lysis by Ly–49$^+$ NK cells (from C57BL/6J [H–2^b] mice). In contrast, H–2^b tumor cell targets are lysed equally well by both Ly–49$^+$ and Ly–49$^-$ NK cells. All targets demonstrating preferential resistance express MHC class I antigens, but several, particularly those of T cell origin, lack MHC class II expression by FACS analysis (not shown). These data suggest that the MHC haplotype of the target cell influences the specificity of Ly–49$^+$ NK cells and raise the possibility that the ligand for Ly–49 is an MHC class I antigen.

To explore this possibility, we examined the R1E/TL8x.1 cell line (H–2^k), which lacks expression of MHC class I antigens because it does not express the β_2-microglobulin gene. The parental cell line, R1.1, is resistant to lysis by Ly–49$^+$ NK cells but is lysed by Ly–49$^-$ NK cells (17). In contrast, the R1E/TL8x.1 cell line is lysed to an equivalent degree by both Ly–49$^+$ and Ly–49$^-$ NK cells (Table 1). These data are consistent with the possibility that the target cell ligand for Ly–49 is an MHC class I antigen.

Studies using target cells susceptible to lysis by both Ly–49$^+$ and Ly–49$^-$ NK cells demonstrate that these targets become resistant to lysis

by Ly–49$^+$ NK cells when they are transfected with H–2D^d, but not with H–2K^d or H–2L^d. The transfected resistance to natural killing also extends to an inability of Ly–49$^+$ effector cells to lyse these targets through other mechanisms including ADCC, lectin-induced cytotoxicity, and mAb-induced redirected lysis (17, 46). Cells transfected with H–2D^d remain susceptible to lysis by Ly–49$^-$ NK cells. Thus, a target that is susceptible to killing by Ly–49$^+$ NK cells is rendered resistant to killing by the selective expression of H–2D^d.

The Ly–49 molecule itself appears to mediate this inhibition of natural killing. Incubation of Ly–49$^+$ NK cells with mAb A1 in the cytotoxicity assay enables the NK cells to lyse the transfected, resistant target (17). Isotype-matched, control mAbs do not affect lysis of these targets by Ly–49$^+$ NK cells even when these mAbs bind to the Ly–49$^+$ effector cells at a density equivalent to that of mAb A1, and mAb A1 does not affect lysis of these targets by Ly–49$^-$ NK cells. Several observations indicate that the ability of mAb A1 to permit target lysis is not due to redirected lysis, a process that requires the presence of Fc receptors on the target cell (47). First, mAb A1 does not induce the lysis of Daudi targets (which express Fc receptors and are susceptible to redirected lysis) (13). Second, several T cell targets that are resistant to lysis by Ly–49$^+$ NK cells and that are killed by the same cells in the presence of mAb A1 do not express Fc receptors, as detected by either mAb 2.4G2 (48) or FITC-IgG staining in FACS analysis (e.g. tumors BW5147, R1.1). Third, protein A has no effect on the ability of mAb A1 to induce lysis of resistant targets, yet protein A blocks redirected lysis (by blocking mAb binding to the target cell Fc receptor) (13). Similarly, F(ab′)$_2$ fragments of mAb A1 are sufficient to induce target cell lysis. Finally, mAbs that induce redirected lysis do not

Table 2 Target cell resistance to lysis by Ly-49$^+$ effector cells by gene transfer and expression of H-2D^d

C1498 cells[a] transfected[b] with	Specific lysis by	
	Ly-49$^+$ NK cells	Ly-49$^-$ NK cells
Wildtype	++[c]	++
D^d	−	++
L^d	++	++
K^d	++	++
Vector DNA	++	++

[a] C1498 cells are H-2^b.
[b] Cells were untransfected (wildtype), or stably transfected with cDNAs encoding MHC class I antigens, or with empty vector DNA as indicated.
[c] See footnote b in Table 1.

induce Ly–49$^+$ NK cells to lyse their resistant targets. These experiments indicate that mAb A1, specific for Ly–49, permits Ly–49$^+$ NK cells to kill resistant targets by a mechanism other than redirected lysis.

Taken together, the data suggest that Ly–49, on the NK cell, interacts with a specific MHC class I antigen on the target cell. The result of this interaction is an inability of the NK cell to lyse its target, possibly through a "negative" signal transmitted by engaged Ly–49 molecules. All known pathways of activating NK cell cytotoxicity appear to be inhibited. Targets that are preferentially resistant to Ly–49$^+$ NK cells cannot be lysed by ADCC or by stimulation of the NK cells either with lectins or with mAbs specific for NK1.1, VEA, or Ly–6 (17, 46). Each of these cytotoxic mechanisms involves the recognition of different molecules on target cells. It seems unlikely that the expression of H–2D^d on the target cell could simultaneously hinder the recognition of all of these target structures. Nor is it likely that the anti-Ly–49 mAb "unmasks" recognition structures on the effector cells. Instead, the effect of mAb A1 can best be explained if Ly–49 delivers an inhibitory signal to NK cells in response to H–2D^d, and this response is blocked by mAb A1. In accord with this hypothesis, a mAb directed against the $\alpha 1/\alpha 2$ domains of H–2D^d also allows lysis to occur, while mAb to the $\alpha 3$ domain does not (17). The same effect is seen using F(ab′)$_2$ fragments of the mAb, indicating that lysis is not due to ADCC. Thus, we currently favor the hypothesis that Ly–49 is an inhibitory receptor for MHC class I antigens.

The mAb blocking studies (17) also suggest that Ly–49 interacts with the $\alpha 1/\alpha 2$ domains of H–2D^d. Because the $\alpha 1/\alpha 2$ domains form the peptide binding cleft of MHC class I molecules, peptides bound to the cleft may influence the interaction between Ly–49 and H–2D^d. Consistent with this possibility are transfection studies (45) demonstrating that a single amino acid mutation (residue 74) in a side pocket of the $\alpha 1$ helix of HLA-A2 converts susceptible target cells to an NK cell–resistant phenotype. The involvement of MHC-associated peptides could explain the susceptibility of the H–2^a (H–2$^{k/d}$ target, YAC–1, that should be resistant to lysis by Ly–49$^+$ NK cells. YAC–1 cells may lack an inhibitory H–2D^d-associated peptide or possess other defects that produce its susceptible phenotype. In the event, it appears that MHC class I molecules may present peptides to NK cells, as they do to T cells.

Relation of Ly–49 To Other Antigens Expressed by NK Cell Subsets

Only a few other antigens have been described that are expressed by distinct NK cell subpopulations. The antigens recognized by mAb GL183

and mAb EB6 are expressed by overlapping subsets of human NK cells (49–51). These mAbs immunoprecipitate structures that appear to be different from mouse Ly–49. They are variably expressed either as a single-chain 58-kDa polypeptide or as a 58-kD polypeptide noncovalently associated with a 55-kDa polypeptide. Their expression correlates with the ability of NK cell clones to lyse certain allogeneic target cells. Moreover, the mAbs affect the cytolytic activity of human NK cell clones against target cells; lysis of certain human tumor targets is enhanced whereas lysis of murine tumors is inhibited. Susceptibility of these targets to lysis has been mapped (52) to the human MHC locus, consistent with the possibility that these NK cell structures may be functionally related to Ly–49.

The mAb SW5E6 precipitates a disulfide-linked dimer (54-kDa subunits) from mouse NK cell extracts. This antigen is expressed by an NK cell subset that appears to mediate rejection of bone marrow cells expressing H–2^d and/or Hh–1^d (53). Expression of the antigen recognized by mAb SW5E6 does not affect lysis of YAC–1 by NK cells. Because the primary structures of the antigens recognized by mAbs CL183, EB6, and SW5E6 have not been defined, their relationship to the Ly–49 antigen remains unclear.

Ly–49, however, is structurally and genetically related to another family of NK antigens, which are expressed on virtually all NK cells and which, in contrast to Ly–49, activate NK cells. These are exemplified by NKR-P1 in the rat, and NK1.1 in mice. Although NK1.1 was the first described, NKR-P1 was the first member of this family to be cloned (54) and is therefore discussed first.

NKR-P1

Structure and Function of Rat NKR-P1

NKR-P1 was first identified on IL–2–activated rat NK cells by the mAb 3.2.3, which identifies all rat NK cells (11). The mAb also reacts, relatively weakly, with polymorphonuclear cells. Antibody to NKR-P1 stimulates degranulation by NK cells. It also mediates redirected lysis, i.e. NK cells can lyse target cells that express the mAb, by attachment to target cell Fc receptors (11). The NKR-P1 surface protein, therefore, has features of an NK receptor: the expression of NKR-P1 is primarily limited to NK cells, and perturbation of NKR-P1 activates cytotoxicity.

In NK cells NKR-P1 also initiates transmembrane signals that mimic the response of these cells to target cells (14, 15, 55, 56). This response resembles the response following perturbation of the TCR on T cells or of immunoglobulin on B cells. In T cells, perturbation of the TCR activates

src-like tyrosine kinases, leading to the phosphorylation of tyrosine residues on distinct proteins, including phospholipase C (57–62). Phospholipase C is thereby activated, resulting in phosphoinositide turnover and a consequent rise in intracellular calcium. NK cells express at least 3 *src*-like tyrosine kinases, lck, fyn and fgr (63, 64) and the interaction of NK cells with target cells activates tyrosine phosphorylation (65, 66) as well as phosphoinositide turnover and a rise in intracellular calcium within NK cells (15, 55, 56). Moreover, inhibitors of tyrosine kinase activity inhibit natural killing (65, 66). Thus, although NK cells have distinct receptors, they may share with T and B cells postreceptor pathways that lead to cell activation, and the activation of these pathways may serve as a marker for putative receptors on NK cells. NKR-P1 is a candidate for such a receptor because antibody to NKR-P1 stimulates both phosphoinositide turnover and a rise in intracellular calcium (14). It also stimulates a pattern of protein phosphorylation similar to that seen following exposure to targets (J. C. Ryan, unpublished observations).

NKR-P1 is expressed on NK cells as a disulfide-linked homodimer of ~60 kDa (11). The NKR-P1 cDNA was cloned by Giorda et al (54), revealing that the protein is a type II integral membrane protein (intracellular amino terminus), with a single transmembrane domain. The cytoplasmic domain is relatively short, consisting of only 39 amino acid residues. The extracellular domain demonstrates features of known C-type (calcium-dependent) lectins, including Ly–49 (18) which, as discussed above, appears to interact specifically with MHC class I antigens. Because many other C-type lectins are cell-surface receptors, the structure of NKR-P1 is consistent with the hypothesis that it serves as a cell-surface receptor, and that its ligand may be a carbohydrate. However, a ligand for NKR-P1 has not yet been identified.

The NKR-P1 Gene Family in Mice

Like Ly–49, NKR-P1 appears to belong to a family of homologous genes. Although Southern blot analysis of rat genomic DNA initially suggested a single gene for NKR-P1 (54), we have identified at least four cross-hybridizing rat cDNAs with related but distinct sequences (J. C. Ryan, unpublished observations). The presence of an NKR-P1 gene family has been more extensively defined in the mouse. Giorda et al used the cDNA for rat NKR-P1 to identify three distinct mouse NKR-P1 cDNAs (67). Although these demonstrate considerable homology in their open reading frames, they have divergent 3′ untranslated regions of different length. Thus the cDNAs are of different sizes, and these correlate with the sizes of three prominent transcripts on Northern blot analysis. This correlation was confirmed by selective hybridization with unique sequences. The three

mouse NKR-P1 cDNAs were initially designated numerically: 2, 34, and 40. For discussion here, we use their Genbank designations: MusNKR-P1A (clone 2), MusNKR-P1B (clone 34), and MusNKR-P1C (clone 40). These are presented in Table 3, where, for reference, they are matched to the numeric designations initially used by Giorda et al and to the numeric designations that we previously assigned to our independently derived clones (12, 18), which are identical or closely related to those of Giorda et al.

As shown in Table 4, rat NKR-P1 is almost equally homologous to MusNKR-P1A and to MusNKR-P1C (72–74% amino acid identity). The 3′ untranslated portion of NKR-P1, however, is more closely related to MusNKR-P1A than to MusNKR-P1C. Moreover, we have recently isolated a rat cDNA from the NKR-P1 family that is more closely homologous to MusNKR-P1C, (J. C. Ryan, W. E. Seaman, unpublished observations), so the mouse homologue of rat NKR-P1 appears to be MusNKR-P1A. MusNKR-P1B is less homologous to rat NKR-P1 (62% amino acid identity).

NK1.1 IS ENCODED BY A MEMBER OF THE MOUSE NKR-P1 GENE FAMILY In certain strains of mice, including C57BL/6, almost all NK cells express the antigen NK1.1 (68, 69), similar to the distribution of NKR-P1 in the rat. Several additional lines of evidence suggested that NK1.1 might be encoded by a member of the mouse NKR-P1 gene family. First, antibodies to NK1.1, like antibodies to NKR-P1 on rat NK cells, activate cytotoxicity (13). Second, NK1.1, like NKR-P1, is a disulfide-linked homodimer (12). Third, as discussed later, the gene for NK1.1 maps to a region now known to encode mouse NKR-P1. Thus, it was surprising that NK1.1 could not be detected on COS cells transfected with MusNKR-P1 cDNAs. It appears now that this lack of expression is unique to COS cells. In particular, expression of MusNKR-P1C in *Sf9* insect cells by infection with baculovirus reveals that this cDNA encodes a protein recognized by a monoclonal antibody (PK136) to NK1.1 (12). NK1.1 can also be expressed by infection

Table 3 Members of the mouse NKR-P1 family

Genbank designation	Designation by Giorda et al	Designation by Yokoyama et al; Ryan et al	Other designation
MusNKR-P1A	2	mNKR-P1.7	
MusNKR-P1B	34	—	
MusNKR-P1C	40	mNKR-P1.9	NK1.1

Table 4 Predicted amino acid sequences of rat and mouse NKR-P1

```
            1                                                   50
RatNKRP1    MD.TARVYLS LKPSKTAAGA QCVSPPSLPP DACRCPRSHR LALKLSCAGL
MusNKRP1A   MD.TARVYFG LKPPRT.PGA WHESPPSLPP DACRCPRSHR SALKLSCAGL
MusNKRP1B   MDSTTLVYAD LNLARI.QEP KHDSPPSLSP DTCRCPRWHR LALKFGCAGL
MusNKRP1C   MD.TASIYLG LKPPRT.LGA WHESPPSLPP DACRCPRSHR LALKLSCAGL

            51                                                  100
RatNKRP1    ILLVLALVGM SILVRVLVQK PSVEPCRVLI QENLSK.TGS PAKLKCPKDW
MusNKRP1A   ILLVVTLIGM SVLVRVLIQK PSIEKCYVLI QENLNKTTDC SAKLECPQDW
MusNKRP1B   ILLVLVVIGL CVLV.LSVQK SSVQKICADV QENRTHTTDC SVNLECPQDW
MusNKRP1C   ILLVLTLIGM SVLVRVLVQK PSREKCCVFI QENLNKTT.. .VNLECPQDW

            101                                                 150
RatNKRP1    LSHRDKCFHV SQTSITWKES LADCGGKGAT LLLVQDQEEL RFLRNLTKRI
MusNKRP1A   LSHRDKCFHV SQVSNTWEEG LVDCDGKGAT LMLIQDQEEL RFLLDSIKEK
MusNKRP1B   LSHRDKCFRV FQVSNTWEEG QADCGRKGAT LLLIQDQEEL RFLLDSIKEK
MusNKRP1C   LLHRDKCFHV SQVSNTWEEG QADCGRKGAT LLLIQDQEEL RFLLDSIKEK

            151                                                 200
RatNKRP1    SSSFWIGLSY TLSDENWKWI NGSTLNSDVL SITGDTEKDS CASVSQDKVL
MusNKRP1A   YNSFWIGLRY TLPDMNWKWI NGSTLNSDVL KITGDTENDS CAAISGDKVT
MusNKRP1B   YNSFWIGLRF TLPDMNWKWI NGTTFNSDVL KITGDTENGS CASISGDKVT
MusNKRP1C   YNSFWIGLRF TLPDMNWKWI NGTTFNSDVL KITGVTENGS CASILGDKVT

            201                         230
RatNKRP1    SESCDSDNIW VCQKELKCEC MCNDS*....
MusNKRP1A   FESCNSDNRW ICQKELYHET LSNYVGYGH*
MusNKRP1B   SESCSTDNRW ICQKELNHET PSNDS*....
MusNKRP1C   PESCASDNRW ICQKELNHET PSNDS*....
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of fibroblasts with retrovirus containing MusNKR-P1C cDNA (J. C. Ryan, unpublished observations).

The identification of NK1.1 as a member of the mouse NKR-P1 family supports a role for NKR-P1 proteins as activating structures on the surface of NK cells. NK1.1 and NKR-P1 represent different members of the family, yet both molecules activate NK cell lysis of target cells.

Deletional Variants of NKR-P1

In their studies of MusNKR-P1 isoforms, Giorda et al, using the polymerase chain reaction, amplified variants that contain deletions within the open reading frame (67). We have found similar variants of Ly–49 (unpublished observations), and our clone of MusNKR-P1C differs from that of Giorda et al by a deletion of nine base pairs (12). Most of the deletions observed in any of these NK surface molecules maintain the same reading frame, suggesting that the variants could produce functional proteins. Giorda et al have recently defined the genomic structure of MusNKR-P1A, and their findings, for the most part, explain its deletional variants by alternate splicing of exons (70).

By whatever mechanism they are generated, the presence of deletional variants could lead to receptor diversity. There is, so far, no evidence for physical rearrangement of MusNKR-P1 or Ly–49 genes. However, there are at least several members in each gene family, and if each of them could generate deletional variants, this would increase the possible repertoire of expression. Currently, this hypothesis remains untested, and the frequency of deletional variants is unknown.

The present evidence suggests that each NK cell expresses more than one member of the NKR-P1 family, perhaps all of them. Thus, NK1.1 is expressed on all NK cells in the mouse, even though the anti-NK1.1 mAb recognizes only the product of MusNKR-P1C, not MusNKR-P1A or MusNKR-P1B (12; and J. C. Ryan, unpublished observations), yet fresh NK cells contain transcripts for all three isoforms. (Antibody 3.2.3, reactive with rat NKR-P1, similarly binds to all NK cells, but it is not yet known if it recognizes only one of the rat NKR-P1 isoforms.) In addition, the rat NK cell line RNK–16 expresses transcripts that correspond in size to the three known mouse transcripts, indicating that different transcripts can be expressed in the same cell. If NK cells express more than one member of the NKR-P1 family, it is possible that heterodimers form between different members of the same family. These have not, however, been demonstrated, nor has it been possible to generate long-term murine NK cell clones in order to examine expression of NKR-P1 transcripts in clonal populations of nontransformed cells.

Expression of MusNKR-P1 in Different Mouse Strains

The NK1.1 antigen is expressed only in certain strains of mice such as C57BL/6, NZB, and CE (68, 69). Although it was initially assumed that NK1.1$^-$ strains might express other alleles of NK1.1, and some have been postulated, no such alleles have been clearly identified. Interestingly, genomic DNA from the strains that express NK1.1 share RFLP variants when probed with mouse NKR-P1 cDNA, while RFLP variants from NK1.1$^-$ strains are different (18, 70). Thus, structural variations in the genome correlate with the expression of NK1.1.

Giorda et al demonstrated by Northern blot analysis with NKR-P1 cDNA, that NK1.1$^-$ mouse strains express very low levels of transcripts for all members of the NKR-P1 family (70). This result does not appear to reflect poor hybridization to alternate alleles, because these investigators have sequenced and compared the genomic DNAs for MusNKRP1A, MusNKRP1B, and MusNKRP1C from NK1.1$^+$ (C57BL/6) and NK1.1$^-$ (BALB/c) mice (70). The genes were highly homologous, demonstrating $>90\%$ identity in the predicted amino acid sequences. There was also 95–98% identity in the nucleic acid sequences from the promoter regions for the three variants. These findings raise the possibility that the expression of all NKR-P1 isoforms is co-regulated.

If strains of mice that lack NK1.1 fail to express all members of the NKR-P1 family, what might be the importance of this gene family in natural killing? Although mouse strains that express NK1.1 tend to have higher levels of natural killing than strains that do not, this is by no means a rule. One possibility is that the NKR-P1 family is only one of multiple related gene families that encode activating structures on NK cells, so that the loss of the NKR-P1 family has only a limited effect on specificity.

Relation of NKR-P1 Sequence to That of Ly–49

Although NKR-P1 and Ly–49 are both disulfide linked homodimers with a type II integral membrane orientation and an external C-type lectin domain, mouse NKR-P1 sequences are only weakly homologous to mouse Ly–49 (18, 67). There is no significant homology at the nucleotide level, and only about 25% identity at the amino acid level. This degree of homology is similar to that between all members of the C-type lectin supergene family. Thus NKR-P1 and Ly–49 appear to represent different families of C-type lectins. This is in accord with their apparent difference in function: Ly–49 inhibits natural killing, whereas members of the NKR-P1 family activate it. This difference in signaling may reflect differences in the cytoplasmic domains of NKR-P1 and Ly–49. There is no significant homology in this region between the two families. In contrast, members

of the MusNKR-P1 family share conserved regions in their cytoplasmic domains including, in particular, the amino acid motif Cys-X-Cys-Pro. This sequence is found also in the cytoplasmic domains of CD8 and CD4, where the cysteine residues have been shown to play a role in the molecular association with the *src*-like tyrosine kinase, $p56^{lck}$ (71). To date, however, no physical association between NKR-P1 and $p56^{lck}$ has been demonstrated, and the cytoplasmic residues of NKR-P1 that may be required for signaling have not been defined.

NKR-P1 Is Genetically Linked to Ly-49: Definition of the Genetic NK Complex (NKC)

Because of the weak homology between NKR-P1 and Ly-49 cDNAs, they do not cross-hybridize. Southern blots of genomic DNA with NKR-P1 or Ly-49 cDNAs nonetheless reveal restriction fragment length polymorphic variants that, when grouped by strain, show overlapping groups (18). These findings suggest that *NKR-P1* and *Ly-49* are genetically linked, a finding we have confirmed by analysis of RFLP variants for *Ly-49* and *MusNKR-P1* in 62 recombinant inbred (RI) mouse strains (18). In previous studies of RI strains (16), we linked *Ly-49* to *Prp* (proline rich proteins), an unrelated gene that has been mapped on mouse chromosome 6 (72). The precise interval between *Ly-49* and *Prp* has not been determined. Comparison of the strain distribution profiles of *MusNKR-P1* and *Ly-49* demonstrate one recombinant in the RI strains examined ($n = 62$), with a calculated (73) recombination frequency that equals 0.0041. In the one recombinant, *Ly-49* remains linked to *Prp*. The discordant mouse confirms evidence from analysis of the cDNAs, indicating that the *Ly-49* and *mNKR-P1* are separable entities. In further support of this, Southern blots of genomic DNA with MusNKR-P1 and Ly-49 cDNA, though showing several bands, show none that are shared (18). *MusNKR-P1* and *Ly-49* therefore represent gene families that are genetically linked, are structurally related, are expressed on NK cells, and are functionally active in NK cell recognition and activation, but which are separate and only weakly homologous to each other.

These gene families are separated by ~0.4–0.5 centiMorgans (Figure 1). Although it is difficult to correlate a physical map distance to genetic linkage data, 1000 kb is approximately equal to 1 cM (74), so that less than 500 kb should separate the Ly-49 and MusNKR-P1 loci. Still, this distance between *MusNKR-P1* and *Ly-49* is considerable, and the NKC may extend beyond these linked loci. Possibly other genes encoding related molecules are included in the NKC, and these may be difficult to detect because they fail to cross-hybridize with Ly-49 or MusNKR-P1 probes. Nevertheless, *Ly-49* and *MusNKR-P1* are anchor loci in a chromosomal

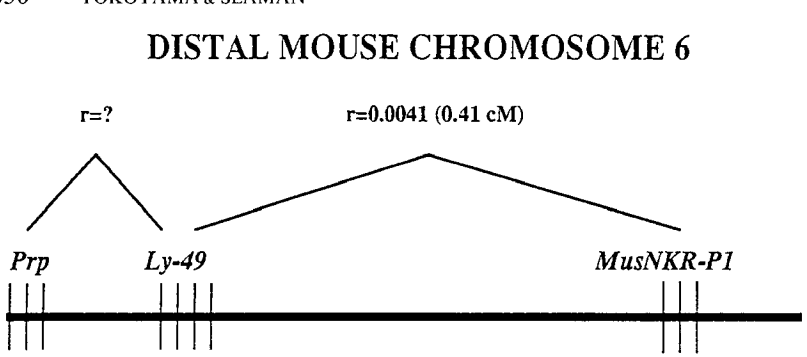

Figure 1 The mouse NK complex. A schematic diagram of distal mouse chromosome 6 is shown. *Prp* is 60 cM from the centromere. The interval between *Ly–49* and *Prp* is not known because no recombinants between these loci have been identified in 63 RI mice. The gene order and number of genes in the Ly–49 and MusNKR-P1 loci have not been determined.

region that contains a series of genes encoding structurally and functionally related cell surface molecules. We have termed this genetic region the NK complex (NKC).

We predict that other molecules encoded in the NKC are type II integral membrane proteins, are normally expressed by NK cells, belong to the C-type lectin supergene family, and are functionally important in NK cell activity. Recent reports of human NK-specific proteins (NKG2) whose structure resembles MusNKR-P1 and Ly–49 are consistent with this hypothesis (75, 76). The human NKG2 molecules are members of the C-type lectin family but are only distantly related to the mouse molecules (75). Although these differences could reflect species differences, the human NK cell molecules also may have mouse homologues in the NKC that are distinct from MusNKR-P1 and Ly–49. Inasmuch as *Prp* lies in the middle of a linkage group conserved between human chromosome 12p and distal mouse chromosome 6 (77), and both *Ly–49* and *MusNKR-P1* are linked to *Prp*, the human homologue of the NKC is likely to reside on chromosome 12p near *Prp* (12p13.2). The recent localization of the genes encoding the NKG2 molecules to human chromosome 12 (76) is consistent with this possibility.

Of great interest with regard to the general function of NKC-encoded products, a single genetic locus, termed *Cmv–1*, encodes resistance of mice to lethal infection with mouse cytomegalovirus (mCMV) (78). Resistance is associated with markedly decreased viral replication in the spleen and has been attributed to the activity of NK cells (79). Since *Cmv–1* is genetically linked to the NKC (78–80), *Cmv–1* might encode an NK cell surface

molecule that is involved in the recognition of mCMV or mCMV-infected cells. Elucidation of the structures and functions of molecules encoded in the NKC should provide significant insights into the mechanisms by which NK cells recognize their targets and are activated.

Overview and Hypothesis: NK Cells Are Activated and Inhibited by Different Receptors Encoded in the NKC

Our studies suggest that the NKC encodes inhibitory receptors (Ly–49), and stimulatory receptors (NKR-P1). NK cells appear to express multiple forms of these molecules, and a single NK cell simultaneously expresses both inhibitory and stimulatory receptors. However, the inhibitory influence of Ly–49 engagement appears to be dominant over any stimulatory signal. We postulate that an NK cell can be activated to kill a target cell if the NK cell expresses an appropriate member of the NKR-P1 receptor family and the target expresses its specific, putative ligand. This does not exclude other activation pathways, and NK cells can also kill targets by ADCC, when immunoglobulin that recognizes a target cell binds to Fc receptors on the NK cells. If the inhibitory receptor is also engaged, however, the activation of NK cells for either cytotoxic pathway is canceled. This most likely occurs by abortion of activation signals at a point distal to the ligand binding of NKR-P1 or Fc receptors. If correct, this regulatory mechanism likely plays a significant role in the NK cell's ability to discriminate between normal and diseased cells. However, the teleology of these recognition events is not yet understood. Further characterization of the structure and physiologic activity of the NKC products will aid our understanding of the molecular basis for NK cell activity and of the expanding role of MHC class I molecules in normal and diseased immune responses, possibly including the association of autoimmune diseases with specific MHC class I alleles.

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GAMMA/DELTA CELLS

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KEY WORDS: $\gamma\delta$ T cells, T cell subsets, thymic selection, T cell development, nonclassical MHC antigens, heat shock proteins, T cell

Abstract

Before TCR rearrangements, T cell progenitors are committed not only to the $\alpha\beta$ and $\gamma\delta$ T cell lineage but also to various subsets of both lineages. In the mouse, distinct $\gamma\delta$ T cell subsets can develop in the fetal thymus, the adult thymus, or independently of a thymus, probably in intestinal epithelia. The two subsets that develop in the fetal thymus home to and are maintained throughout adult life in the skin and the mucosa of the uterus, vagina, and tongue. They are monospecific. This unusual restriction in receptor repertoires is the result of severe limitations in the generation of diversity in the fetal progenitors of these subsets and the thymic selection. After birth, one $\gamma\delta$ T cell subset appears in the blood, spleen, and lymph nodes and one in the intestinal epithelia. The receptor repertoires of these subsets are characterized by the preferential usage of particular Vγ gene segments and extensive junctional diversity. Several murine and human $\gamma\delta$ T cell clones have been shown to recognize classical MHC class I and class II proteins or MHC class I–like proteins, and in very few cases the presented peptides are known. We suspect that the various murine $\gamma\delta$ T cell subsets interact with different antigen presenting cells which utilize different antigen presenting proteins and reside in different tissues. The function of $\gamma\delta$ T cells remains unknown. Preliminary results of experiments

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with gene knock out mice which lack either $\alpha\beta$ T cells or $\gamma\delta$ T cells or both suggest that $\gamma\delta$ T cells do not function as helper cells in humoral immune responses but may complement $\alpha\beta$ T cells in the defense against various microorganisms.

INTRODUCTION

The long odyssey of immunologists searching for the T cell receptor for antigen (TCR) came to an end in 1984 when clonotypic antibodies were raised against T cell clones, and genes rearranging in T cells but not in B cells were identified and cloned. Because the clonotypic antibodies that were likely to recognize idiotypic determinants of the TCR precipitated an $\alpha\beta$ heterodimer (1–5), it came as a surprise when the third rearranging gene called γ was found (6, 7). Antibodies raised against peptides that were synthesized according to the sequence of the γ gene revealed a second TCR heterodimer, $\gamma\delta$ (8). The δ gene was cloned owing to its location within the TCR α locus (9). Further studies showed that cells expressing $\gamma\delta$ heterodimers did indeed exist and represented a new T cell class (10–13). Thus the immunology of $\gamma\delta$ T cells has been progressing in a "reversed direction" i. e. from genes to the TCR and from the TCR to a new class of lymphocytes. At present many laboratories are attempting to elucidate the functions of these cells.

Previous reviews have focused on the structure and organization of γ and δ genes as well as on the development and specificity of $\gamma\delta$ T cells in mice (14–18), human (17–21), and other species (22). In the present review we focus on the specificity and function of $\gamma\delta$ T cells.

MOUSE $\gamma\delta$ T-CELLS

Segregation of $\alpha\beta$ and $\gamma\delta$ Lineages

In the fetal thymus, rearrangements and surface expression of γ and δ genes precede those of α and β genes (23, 24). This and the finding that nonproductive rearrangements of γ genes are common in $\alpha\beta$ T cells (25) led to the belief that $\alpha\beta$ T cells are derived from T cell progenitors that failed to express $\gamma\delta$ TCRs (24). If this was correct $\alpha\beta$ T cell development should be impaired in transgenic mice expressing rearranged γ and δ transgenes. However, in the first $\gamma\delta$ TCR transgenic animals $\alpha\beta$ T cells developed normally (26). No transgenic transcripts were found in the $\alpha\beta$ T cells of these mice. Silencing of in-frame and out-of-frame rearranged γ genes was also observed in $\alpha\beta$ T cells of normal mice (27). A second set of $\gamma\delta$ TCR transgenic mice was generated with shorter transgenes which

apparently lacked the silencer element (28). In these mice αβ T cell development was severely retarded. These findings suggest that it is the expression of a γ silencer that determines the commitment to the αβ T cell lineage.

A similar silencing element is associated with the TCR α gene (29). Since the γ gene rearranges before the α gene, the α gene silencer cannot be primarily involved in the αβ versus γδ lineage commitment. Perhaps it determines the temporal order of β and α gene rearrangements in cells that have already been committed to the αβ lineage by the previous expression of the γ silencer.

According to another model the deletion of the δ locus by a novel recombination is a prerequisite of αβ T cell development (30, 31). However the normal development of αβ T cells in γδ TCR transgenic mice is not readily compatible with this proposition.

γδ T Cell Subsets

In mice there are various subsets of γδ T cells, which differ from each other by parameters such as time of appearance in ontogeny, anatomical location, TCR repertoires, and thymus dependence (see Table 1). According to the Vγ segments used preferentially or exclusively, we refer to these subsets as the V5, V6, V4 and V7 subsets. Each subset must express unique adhesion proteins that are responsible for the differences in their migratory behaviour. Two monospecific subsets are disseminated in epithelia, the V5 subset in the epidermis of the skin (32) and the V6 subset in the mucosal surfaces of the uterus, vagina, and tongue (33). They are both derived from the γδ T cells which appear first in the fetal thymus (34–37). Soon after birth most of the γδ T cells in the thymus express the Vγ4 and Vγ1 gene segments and a few of them express Vγ2 and Vγ7 (36, 38, 39). These γ chains pair with many different δ chains, and both chains exhibit great junctional diversity (38, 40, 41). Like αβ T cells V4 subset cells circulate through blood and lymphoid organs such as spleen and lymph nodes (39). It is not clear, however, whether γδ T cells recirculate from blood to lymph in lymph nodes. Indeed a histological study of sheep lymph nodes suggests that γδ T cells do not recirculate from blood to lymph and that they have no functional role in lymph nodes (22, 42).

One subset of γδ T cells is generated outside the thymus, probably somewhere in the intestinal epithelia (43–46). These cells appear during the first weeks of life (43). Unlike thymus dependent lymphocytes but similar to thymus independent αβ T cells, most of these cells do not express Thy1 and do express CD8α homodimers (43, 46, 47). Their TCR repertoire is characterized by the predominant usage of Vγ7 and Vγ1 chains, expression of multiple Vδ chains, and high junctional diversity (48–50; P.

Table 1 Mouse $\gamma\delta$ T cells

Subset	Location	TCR usage	Diversity	Characteristics
V5	Skin	V5JIC1-γ V1D2J1C-δ	None	Generated in 14–17 day-old fetal thymus Generation depends on fetal progenitors and fetal thymus Positive selection of monospecific cells in the thymus Homing to and maintenance in epidermis Recognizes cultured keratinocytes
V6	Vagina, uterus, tongue	V6J1C1-γ V1D2J1C-δ	None	Generated in late fetal and newborn thymus Positive selection of monospecific cells in the thymus Homing to and maintenance in mucosal epithelia (vagina, uterus, tongue)
V1	Spleen, intestine, skin	V1J4C4-γ V6, 4, 5, 7-δ	High	Abundant in newborn thymus and in spleen 1–2 week after birth Autoreactive, spontaneous IL2 production Respond also to mycobacterial Hsp60 and Hsp60 peptide 180–196 Autoreactivity and response to exogenous Hsp is blocked by mAb to $\gamma\delta$ TCR, VNR but not class I or II MHC proteins Various effects on hemopoesis in transgenic mice
V4	Blood, lymph nodes, spleen	V4J1C1-γ V5, 4, 6, 7-δ	High	Generated in postnatal thymus Major $\gamma\delta$ cell population in adult thymus, lymph nodes and spleen
V7	Intestine	V7J1C1-γ V, 4, 5, 6, 7-δ	High	Highly diversified Thymus independent Expression of CD8α homodimers
	Lung	Vγ4 Vδ6	High	8–20% of resident pulmonary lymphocytes are $\gamma\delta$ T cells
	Liver	V1, 2-γ Vδ6	High	Number increases with age Express CD8α homodimers
	Mammary gland	V4, 5-γ Vδ4	High	Fourfold increase of number in lactating mammary gland

Pereira, unpublished observations). In Table 1 we included subset V1 because of its unique autoreactivity even though V1 is probably not a distinct subset, i.e. it probably does not have a distinct progenitor but is rather a population derived from different progenitors but with common properties and Vγ usage. The V1 population has a thymus dependent and independent component.

The γδ T cell populations in the liver, lung, and mammary gland also cannot readily be assigned to one or the other of the above described subsets (51–53). They also may represent mixtures of cells from different subsets.

Human γδ T cell subsets are listed in Table 2. They are described in a later section.

Heterogeneity of Subset Progenitors

We assume that all the γδ T cell subsets described above are derived from different progenitor cells that are committed to give rise to distinct sublineages before any TCR gene rearrangements have taken place. This belief is based mainly on two sets of data.

First, γδ T cell subset progenitors differ in their requirements to generate mature progeny. One requires a fetal thymus, one requires an adult thymus, and one does not require any thymus to generate mature progeny. The V7 subset is thymus independent because it is found in the intestines of athymic nude mice and in thymectomized mice that have been lethally irradiated and reconstituted with syngeneic bone marrow cells (43, 46). A fetal thymus is required for the generation of the V5 and V6 subsets. An adult thymus fails to support the generation of the fetal subsets but is sufficient for the generation of the V4 and V1 subset (54, 55).

Table 2 Human γδ T cells

Subset	Location	TCR usage	Diversity	Characteristics
Vδ1	Thymus	VIC2-γ Vδ1 most non S-S	High	Predominant in thymus Rare in blood Proportion in blood decreases with age Most cells remain CD45RA
Vδ2	Blood	V2C1-γ Vδ3 most S-S	High	Rare in thymus Predominant in blood Proportion in blood increases with age Most cells become CD45RO

Second, $\gamma\delta$ T cell subset progenitors are committed to different modes of TCR rearrangements. Rearrangments may be "targeted" to different variable gene segments in different progenitors. Thus, a limited analysis of nonproductive rearrangements in mature cells expressing either Vγ4 (56, 57) or Vγ5 (32) chains revealed a preference for rearrangements of Vγ4 or Vγ5, respectively. Similarly, PCR-aided Southern blot analysis of Cγ1 cells colonizing the intestinal epithelia showed a strong bias for the rearrangement of Vγ7, both in the expressed and in the nonexpressed chromosome (49). Furthermore, in cultures of day–13 fetal liver cells with day–14 fetal thymus stromal cells, the temporal order of Vγ gene rearrangements corresponded to the temporal order of the appearance of the Vγ5 and Vγ6 expressing cells during thymic ontogeny (58). It should be noted that in one study (59), PCR analysis of Vγ gene rearrangements in early fetal thymocytes did not reveal any restricted use of particular Vγ gene segments. However, the data presented in this report are not quantitative. The demonstration that fetal precursors have the potential to rearrange any Vγ gene does not argue against the notion of "targeted rearrangements" i.e. preferential rearrangements of defined Vγ genes. Moreover, the rearrangements observed in this study may have occured not only in $\gamma\delta$ but also in $\alpha\beta$ T cell progenitors in which no particular $\gamma\delta$ T cell differentiation program is established.

In fetal progenitor cells the rearrangements are not only limited by a restricted usage of variable gene segments but also by the preferential joinings of segments with short sequence homologies and by the lack of N-region additions. The genes that are assembled in fetal progenitor cells appear to encode receptors that mediate "the conservative view of the immune system" with particular recognition of "old" self and microbial antigens. A very similar distinction can be made between fetal and adult subsets in the other two major classes of lymphocytes, B-cells (60–64) and $\alpha\beta$ T cells (65–67).

The different modes of rearrangement in different $\gamma\delta$ progenitor cells are part of a coordinated differentiation program in which the expression of a particular TCR-repertoire is linked to functional properties such as homing to and maintenance in different peripheral tissues. The homing to different epithelia appears to be independent of the TCR. This has been demonstrated for two $\gamma\delta$ T cell subsets in TCR transgenic mice in which T cells expressing "wrong" TCR's were found in the skin and the intestine (68). However, detailed studies of the lifespan and turnover of $\gamma\delta$ T cell populations in normal and $\gamma\delta$ TCR transgenic mice are necessary to examine the role of the $\gamma\delta$ TCR in the maintenance of the subsets in their "home" tissues. In the case of the fetal subsets, it is conceivable that continuous recognition of a self-antigen is required at least for their main-

tenance in the epithelia of the skin and the mucosae of uterus, vagina, and tongue.

Thymic Selection

The finding that the junctional sequences of rearranged Vγ5, Vγ6 and Vδ1 genes in PCR amplified DNA of fetal thymocytes and epidermal γδ T cells showed a limited junctional diversity in nonproductive rearrangements but almost none in productive rearrangements suggested that the accumulation of cells expressing the invariant Vγ5Vδ1 and Vγ6Vδ1 TCRs was due to TCR-mediated positive selection (32, 37). The monospecificity of the fetal subsets also could be the result of molecular constraints at the level of rearrangements and/or assembly of the heterodimeric TCR molecules. Several efforts were made to determine whether the "cellular selection model" or the "molecular constraint model" or both were correct. Receptor-mediated positive selection is best demonstrated by changing or removing the selecting ligand. Thus, several different mouse strains were analyzed in the hope of finding a polymorphism of the putative selecting ligand and consequently a different canonical sequence. The canonical sequences were the same in all strains tested (J. Lafaille, S. Tonegawa, unpublished). In an attempt to artificially alter the selection process Itohara & Tonegawa added antibodies against a constant region of the γδ TCR to fetal thymus organ cultures in which the monospecific fetal γδ T cell subsets are normally generated (58). The addition of the antibodies led to an increase in the frequency of productive Vγ5, Vγ6, and Vδ1 rearrangements with noncanonical junctional sequences. This finding supports the selection model because it shows that the molecular constraint model alone cannot explain the TCR homogeneity of the two fetal subsets.

Recently the same investigators produced mutant mice with a large deletion of the Cδ gene (S. Itohara, P. Mombaerts, J. Lafaille, A. Nelson, A. Farr, S. Tonegawa, submitted). Since these mutant mice do not express γδ TCR on their surface but do undergo Vγ-Jγ and Vδ-D-Jδ rearrangements, they are ideal to address the molecular constraint vs the cellular selection model. Surprisingly the canonical TCR genes that were assembled in mutant mice in the absence of TCR-mediated selection were as homogenous as those assembled in wild type mice. Indeed the preferred joins seem to be generated by using short sequence homologies present at the borders of the gene segments or in the so-called P nucleotides (37). This finding strongly supports the molecular constraint model. It also suggests that the effect of the anti-γδ TCR antibodies on the junctional diversity of the canonical TCRs in the fetal organ cultures was not due to inhibition of positive selection. We assume that the antibody led to the expansion of very rare cells expressing noncanonical TCRs while, for reasons which we

do not understand, it had little or no effect on cells expressing canonical TCRs, which presumably encountered endogenous ligands in the cultures.

Thus the unusual homogeneity of the TCR of the Vγ5 and Vγ6 subsets appears to result from three processes, namely, rearrangement of specific V segments (i.e. targeted rearrangements), rearrangements guided by short homologous regions at the break points (molecular constraint model) and by positive selection (cellular selection model).

The analysis of γδ TCR transgenic mice provided evidence for positive and negative selection of cells belonging to the adult V4 subset. Negative selection was demonstrated in two studies (69, 70). In the first study the γδ TCR transgenes were derived from the KN6 hybridoma which recognizes the T22 gene product (71, 72) expressed by spleen cells from H–2^b mice but not H–2^d mice (which carry a nonfunctional T22 gene) (72). The number of cells expressing KN6 TCR was similar in the thymus of transgenic H–2^b and of H–2^d mice, but 10 times lower in the spleens of H–2^d mice. Thymocytes and spleen cells from KN6 TCR ligand-positive H–2^b mice were anergic in that irradiated H–2^b spleen cells failed to induce them to produce IL–2 but did induce them to proliferate if exogenous IL–2 was added (69).

In the second study the γδ transgenes were derived from the G8 γδ T cell clone that was obtained from BALB/c nude mice and that is specific for a TL region encoded protein similar if not identical to the KN6 TCR ligand (73, 74). Transgenic TCR expressing cells were found in the intestines of TCR transgenic H–2^b mice, but not in their peripheral lymphoid organs such as lymph nodes and spleen (70, 75). The intestinal cells were unresponsive to H–2^b stimulator cells even in the presence of exogenous IL–2. The anergic state was suspected to be followed soon by apoptosis, but this remains to be demonstrated (75).

Evidence for positive selection of γδ T cells was obtained when the two TCR transgenic mice described above were crossed with β2m deficient mice (76, 77). Cells expressing the transgenic TCR at high levels were abundant in the thymus of β2m-deficient H–2^b and H–2^d TCR transgenic mice but did not exit to peripheral lymphoid tissues and did not give a strong proliferative response to H–2^b spleen cells even when exogenous IL–2 was added. The proportion of transgenic TCR expressing thymocytes that are stained with J11d antibodies was nearly 100% in mice with the β2m defect but only 50% in mice without this defect. These findings suggest that the maturation of transgenic γδ TCR expressing cells is arrested in the thymus of β2m deficient mice. J11d appears to be a marker for immature thymocytes not only in the αβ but also in the γδ T cell lineage. After emigration from the thymus the expression of J11d appears to be lost in both lineages (K. A. Kelly, M. Pearse, L. Lefrancois, R. Scollay, unpublished observations).

Not all γδ T cells require positive selection by β2m dependent proteins since no gross abnormalities of γδ T cells were observed in β2m deficient mice that were not transgenic for a particular γδ TCR (78). This finding does not exclude the possibility that most γδ T cells have β2m related specificities and depend on selection by β2m-associated proteins since a few γδ T cell clones with β2m unrelated specificities could expand in the β2m deficient mice to fill up the γδ T cell compartment.

The data obtained with the KN6 TCR transgenic mice and their crosses with the β2m-deficient mice suggest that positive and negative selection are mediated by the recognition of different ligands because only the former is seen in H–2^d mice (72). We propose that the positively selecting ligand in H–2^b mice is the $T22^b$ protein and in H–2^d mice the product of the T10 gene, a highly homologous duplicate of the T22 gene. The $T22^d$ allele is known to be defective. According to this hypothesis, induction of anergy and activation of mature cells requires recognition of the $T22^b$ protein plus a peptide that cannot be presented by the T10 protein.

Extrathymic Selection of γδ T Cells

Two groups found that the frequency of cells expressing γ or δ chains with particular sequence motifs varied greatly in different strains of mice. Thus, Sim & Augustin have shown that two TCR sequences named BID and GxYS were expressed by many pulmonary resident lymphocytes from BALB/c mice and BALB.B mice but not from C57BL/6 mice (79–81). The same sequences were also found in (BALB/c × C57BL/6) F1 hybrids and in athymic BALB/c mice. The lack of cells expressing BID TCRs and GxYS TCRs in the lungs of C57Bl/6 mice is not due to a failure of these mice to generate the corresponding γ and δ chain genes, because these genes were found in the thymus of all mouse strains (80). These results suggest that the cells expressing BID TCRs were positively selected by strain specific polymorphic ligands that are encoded outside of the classical H–2 region. The selection can take place in the absence of a thymus.

Lefrancois et al (45) reported that the frequency of lymphocytes expressing the Vδ4 chain in the intestinal γδ T cell population varies from 20% to 50% in different strains of mice. F1 hybrids between Vδ4 high and low expressors were Vδ4 high expressors. The analysis of normal and thymectomized F1 into parent bone marrow chimeras showed that the Vδ4 high expressors were selected by host cells and that a thymus was not required for the selection. Further analysis of recombinant inbred strains and of mice recombinant within H–2 suggested that the Vδ4 high phenotype was controlled by a gene linked to the class II MHC genes and required I-E expression.

In these examples of extrathymic selection it is not clear whether exo-

genous antigens are involved nor whether the selection acts upon immature or mature cells. The most likely explanation seems to us to be antigen driven expansion of mature cells.

Figure 1 gives an overview of $\alpha\beta$ and $\gamma\delta$ T cell development.

$\gamma\delta$ T-CELLS IN OTHER SPECIES

$\gamma\delta$ T cells have been found in all vertebrates examined so far, including humans (12, 13), chickens (82, 83), rats (84, 85), sheep (22), cattle (42),

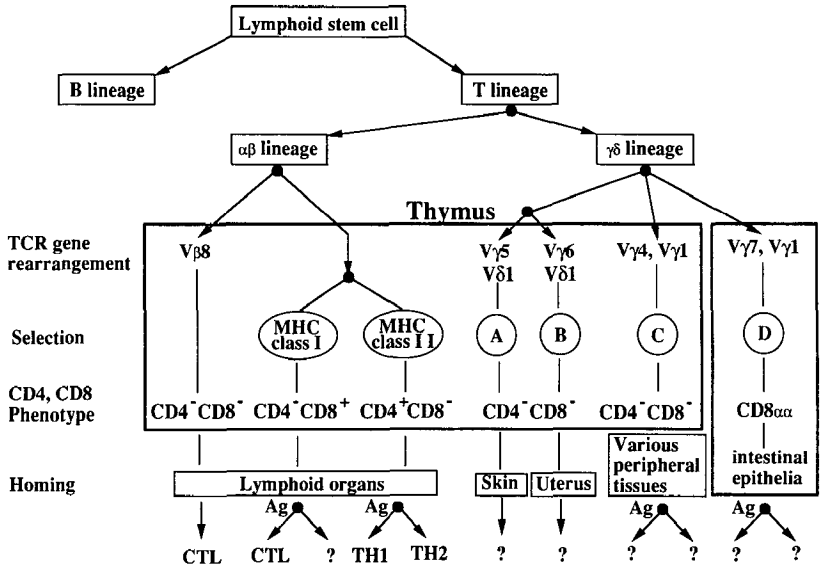

Figure 1 The commitment to functionally distinct subsets (marked by a black dot) may occur at all stages of differentiation, namely before TCR expression, after TCR expression but before maturation, and after maturation. Before TCR expression commitment is made not only to $\alpha\beta$ T-cells and $\gamma\delta$ T cells but also to subsets of both classes, namely, fetal subsets with restricted V segment usage (targeted rearrangements) and very limited junctional diversity and adult subsets with extensive combinatorial and junctional diversity. Positive selection of immature $\alpha\beta$ T cells in the thymus is associated with the commitment to the functionally distinct CD4 and CD8 $\alpha\beta$ T cell subsets that is most likely instructed by TCR recognition of class II and class I MHC proteins, respectively. There is also evidence for positive selection of $\gamma\delta$ T cells in the thymus. We assume that the selecting ligands of the various subsets are nonpolymorphic proteins such as TL region encoded proteins which we arbitrarily call A, B, C and D. Some $\gamma\delta$ T cells may also be selected by classical MHC proteins. After maturation different modes of antigenic stimulation can induce functionally distinct $\alpha\beta$ CD4 T cell subsets, namely TH1 and TH2 cells. Mature $\gamma\delta$ T cells resemble mature $\alpha\beta$ T cells in that they can lyse target cells and secrete the same lymphokines. The functions of the various $\gamma\delta$ T cell subsets remain to be elucidated.

and pigs (86, 87). (Table 3). A preferential localization to epithelia has been noticed in all these species, but there are differences in the abundance of cells and tissue distribution. Ruminants for example have more γδ T cells than αβ T cells in the blood (22, 42). In human (88, 89) and chicken (83), there seems to be no special γδ T cell population in the epidermis.

Human γδ T cells have been studied extensively with regard to TCR repertoire and putative sublineages. Rearrangements at the human TCR γ and δ loci also appear to occur in a developmentally ordered fashion (90, 91). The γδ TCR repertoire that is initially generated in the fetal thymus is small because of the targeting of rearrangements to a limited number of variable gene segments and because of very limited junctional diversity. In the thymuses of 8.5- to 15-week-old human embryos, rearrangements involve joinings of Vδ2 to Dδ3 and of Vγ1.8 or Vγ9 to the Jγ1 cluster (90, 91). The cells which express these TCR chains may be referred to as the Vδ2 subset. From 4 to 6 months after birth, rearrangements involve joinings of other Vδ segments, in particular Vδ1 to Dδ1 and Dδ2 and joinings of upstream Vγ gene segments in the Vγ1 family including Vγ2, 3, 5 and 8 to the Jγ2 cluster (90, 91). The cells which express these TCR chains may be referred to as the Vδ1 subset. The TCR chains of this subset exhibit extensive junctional diversity.

The two human γδ T cell subsets can be distinguished by monoclonal antibodies such as δTCS1 which recognizes Vδ1Jδ1 and Vδ1Jδ2 but not Vδ1Jδ3 (92), BB3 which recognizes Vδ2 (93) or TiγA which recognizes Vγ9 (94). In the postnatal thymus the Vδ2 subset represents about 15% and the Vδ1 subset about 80% of all γδ T cells (95, 96). These proportions of γδ TCR expressing thymocytes remain relatively constant throughout adult life (96). In the blood, however, the Vδ2 subset increases with age from about 25% in cord blood to more than 70% in the blood of most adults. The Vδ1 subset decreases from about 50% in cord blood to less

Table 3 Distribution of γδ T cells (% of lymphocytes in each organ). Data compiled from: humans (88, 89); mice (33, 36); rat (84, 85); chicken (82, 83); sheep (22) and cattle (42)

	Humans	Mice	Rat	Chicken	Sheep	Cattle
Blood	0.5–16	0.5–2	2	15	15–50	15–40
Thymus		0.5–1.5		10	1–4	1–5
Spleen	2–30	0.5–2	2	25	5–7	
Lymph node	5	0.5–3	4		1–6	1–3
Intestine	10	50	−	+	+	+
Skin (epidermis)	−	+		−	−/+	+
Other epithelia (tongue, etc.)		+			+	+

than 30% in the blood of adults (95–99). Most Vδ2 subset cells become positive for CD45RO, a probable marker for memory cells, while most Vδ1 subset cells remain CD45RO negative (100–102). The accumulation of CD45RO positive Vδ2 cells in the blood is thought to be the result of stimulation of mature cells by common ligands for Vδ2/Vγ9 TCRs, many of which are suspected to be superantigens (96). Selection of the predominant γδ T cell subset in adult human blood by superantigens is consistent with the extensive junctional diversity of their TCR (103).

γδ T-CELL SPECIFICITY

Self-Antigens

The murine V5 subset recognizes cultured keratinocytes or fibroblasts treated with tryptic digests of keratinocytes (104). Since the third complementarity determining regions of the canonical TCR of the V6 and V5 subset are identical one might speculate on the basis of current models of TCR/antigen/MHC protein interactions that the two TCRs recognize the same endogenous peptide in the context of different tissue specific peptide presenting proteins. The presenting proteins appear not to be classical MHC proteins.

The V1 population also appears to recognize an endogenous antigen that is expressed by lymphocytes and probably other hemopoietic cells. Since most autoreactive cells of the V1 subset also recognize heat shock proteins they will be described below.

Cultured human γδ T cells often lyse autologous target cells. However, this killing does not involve the γδ TCR. In most cases it is due to IL–2 induced promiscous killing activity that has also been observed with many IL–2 dependent αβ T cell clones. The biological significance of this undiscriminating lytic activity in vitro is questionable.

Classical MHC Proteins

A few murine and human γδ T cell clones have been shown to be specific for class I and class II proteins (Table 4). MHC class I and class II protein specific γδ T cell clones were obtained from nude mice after immunization or repeated in vitro stimulation with allogeneic spleen cells (74, 105, 106). The specificity of these clones was unusual in that it was broadly cross-reactive for the products of different alleles. Several human γδ T cell clones were shown to recognize HLA-A2, HLA-A24, HLA-DR7, HLA-DR3 (107–110) or HLA DQA1/DQB1 heterodimers (111).

Recognition of MHC proteins by γδ T cells probably involves recognition of presented peptides. In one recent study four human clones have been shown to recognize HLA DRw53 and tetanus toxin peptide 1235–

Table 4 Recognition of classical MHC proteins by γδ T cells

Origin of γδ T cells	Specificity	References
$CD4^-$ $CD8^-$ γδ T cell line obtained from draining lymph node cells of C57BI/10 mice 7 days after immunization with B10.BR spleen cells in CFA in footpat	H-2 D^k (+peptide?)	Bluestone et al (1988) *J. Exp. Med.* 168, 1899
Lymph node cells from C57BI/10 nude mice were repeatedly restimulated in vitro with B10.BR spleen cells, cloned and fused with BW5147 thymoma cells; hybridoma LBK.5F3	E^k, E^b, E^s, E^p (+peptide?)	Matis et al (1989) *Science* 245, 746
γδ T cell clones were obtained from cultures containing purified $CD4^-$ $CD8^-$ PBL from healthy donors and allogeneic stimulator cells; Clone 40.1(Vδ1)	HLA-A24 (+peptide?) lysis of P815 cells transfected with A24 cDNA	Ciccone et al (1989) *Eur. J. Immunol.* 19, 1267
γδ T cell clones were obtained from cultures containing purified $CD4^-$ $CD8^-$ PBL from healthy donors and allogeneic stimulator cells; Clones ES-204 (Vδ3) and ES-443 (Vδ1)	HLA-A2 (+peptide?) recognition of P815 cells transfected with A2 cDNA	Spits et al (1990) *J. Immunol.* 144, 4156
γδ T cell clones were obtained from cultures containing purified $CD4^-$ $CD8^-$ PBL from healthy donors and allogeneic stimulator cells after repeated restimulations and cell separations	HLA-DR1 (0501)/HLA DQB1 (0301) *cis* or *trans* encoded heterodimer (+peptide?)	Bosnes et al (1990) *Eur. J. Immunol.* 20, 1429
Two donor specific γδ T cell clones from mitogen stimulated PBL of a patient with a HLA mismatched kidney graft; clone 21 and 40	HLA-DQw6 HLA-A24	Vandekerckhove et al (1990) *J. Immunol.* 144, 1288
Four γδ T cell clones isolated from cultures containing the synovial fluid of T cells of rheumatoid arthritis patients that were repeatedly restimulated with AP-MT in the presence of autologous PBMC	HLA-DRw53+ tetanus toxin peptide (1235–1246); also reactive to AP-MT without DR restriction	Holoshits et al (1992) *J. Clin. Invest.* 89, 308
γδ T cells were isolated from blood and clones were established by stimulation with irradiated allogeneic PBMC, EBV-B cell line, PHA and IL2; clone N2A11	HLA-DR7 (+peptide?)	Jitsukawa et al (1988) *Eur. J. Immunol.* 18, 1671

1246 (112). These examples of $\gamma\delta$ T cell specificity for classical MHC proteins are exceptions. In many cases $\gamma\delta$ T cell responses could not be inhibited by antibodies against the classical MHC proteins that serve as restriction elements for $\alpha\beta$ T cells. Moreover, large numbers of murine $\gamma\delta$ T cell hybridomas failed to recognize classical H–2 proteins (113), and the vast majority of human $\gamma\delta$ T cell clones that were activated in limiting dilution cultures were not specific for the HLA proteins of the stimulator cells (114). Clearly, $\gamma\delta$ T cells do not have the bias for classical class I or class II MHC proteins that is characteristic for $\alpha\beta$ T cells.

MHC-Like Proteins

TL region encoded proteins are not only expressed by thymocytes and leukemic T cells but also by epithelial cells in the intestine. The $T3^b$ gene of C57Bl/6 mice and the $T3^d$ and $T18^d$ genes of BALB/c mice are highly expressed in the epithelium of the small intestine (115). A $T3^b$ product-specific antibody binds to columnar epithelial cells (116) which are in close contact with intestinal $\gamma\delta$ T cells (117). Two other TL region genes of C57BL/6 mice, $T9^b$ and $T21^b$ are also expressed almost exclusively by intestinal epithelial cells (116). The various murine $\gamma\delta$ T cell subsets may possibly recognize antigens that are presented by different tissue-specific TL region–encoded proteins (72, 118). However, thus far only a very few murine $\gamma\delta$ T cell clones have been shown to recognize TL region–encoded proteins (Table 5). The hybridoma KN6 mentioned previously is specific for the $T22^b$ gene product (72, 119). TL region–encoded proteins may serve as antigen-presenting molecules. Indeed, recently Imani et al (120, 121) have been able to show the binding of two peptides to the $T23^b$ (Qa–1^b) protein and one hybridoma recognizes the $T23^b$ protein and one of these peptides, namely the synthetic copolymer Glu: Tyr (122).

The human MHC class I family includes at least 15 loci other than the classical transplantation antigens HLA-A, B, and C (123), many of which may be located telomeric of the HLA-A locus (124). Some of these genes encode class I like proteins which may be the human equivalents of the murine TL region encoded proteins (125). Recognition of these proteins by $\gamma\delta$ T cells has not been described. However, a hint for the recognition of human MHC class I–like proteins was obtained when $\gamma\delta$ T cells from peripheral blood were stimulated with a HLA loss variant cell line (126). Cytolytic T cells were generated in this culture that specifically lysed the HLA loss variant cells. The killing was inhibited by anti-$\gamma\delta$ TCR antibodies and by antibodies against HLA-B and C even though HLA-B and C proteins were not expressed. A similar inhibition pattern was also shown for a $\gamma\delta$ T cell clone, the specificity of which could not be mapped to classical class I MHC genes (127). These findings were interpreted to mean

Table 5 Recognition of MHC like proteins by γδ T cells

Origin of γδ T cells	Specificity	References
γδ T cell clones obtained from draining lymph node cells of Balb/c nudes 7 days after immunization with B10.BR spleen cells in CFA in foot pat; clones FY and G8	TLa[a]? (+peptide?)	Bluestone et al (1988) *J. Exp. Med.* 168, 1899
γδ T cell hybridoma obtained by fusion of $CD4^-$ $CD8^-$ thymocytes of adult C57Bl/6 mice with BW 5417	H-2 T22[b] (+peptide?)	Bonneville et al (1989) *PNAS* 86, 5928 Itoh et al (1990) *Cell* 62, 549 van Kaer et al (1991) *Immunol Rev.* 120, 8
γδ T cell hybridoma obtained by fusion of BW5147 with draining lymph node cells from DBA/2 mice immunized 7 days previously with the synthetic copolymer Glu:Tyr (GT)	H-2 T23[b](Qa-1[b], 37)+GT	Vidovic et al (1989) *Nature* 340, 646
Intestinal γδ IEL	T3?	Kronenberg, pers. commun.
γδ T cell line (IPD2), Vγ9/Vδ1 obtained from immunodeficient patient	CD1c (+peptide?) lysis of human rhabdomyosarcoma cell line transfected with CD1c cDNA	Porcelli et al (1989) *Nature* 341, 447
γδ T cell line (J287), Vγ3 or 4/Vδ1 isolated from blood of healthy donor	CD1c (+peptide?) (rare specificity)	Faure et al (1990) *Eur. J. Immunol.* 20, 703
γδ T cell clones were obtained from PBL of two healthy donors (E and G) stimulated with allogeneic B-LCL	T cell target 1 (TCT.1 = Blast-1 = CD48) (+peptide?) CD48 is a member of Ig superfamily and is encoded in the same band of chromosome 1 as the CD1 gene cluster	Mami-Chouaib et al (1990) *J. Exp. Med.* 172, 1071 Del Porto et al (1991) *J. Exp. Med.* 173, 1339 Mami-Chouaib et al (1991) *J. Immunol.* 147, 2869
One clone isolated from PBL after stimulation with allogeneic B-LCL and IL-4	Class I like MHC antigen	Spits et al (1989) *J. Immunol.* 143, 1506
Two clones isolated from PBL after stimulation with HLA class I loss variants	MHC class I like protein reactive with anti-HLA B and HLA-C antibodies (+peptide?)	Lam et al (1990) *J. Immunol.* 145, 36

that the γδ T cells recognized a peptide presented by MHC class I like proteins that crossreact with antibodies against HLA-B and C proteins.

Two human γδ T cell clones were found to recognize CD1c (128, 129), which is encoded by one gene of a cluster of five closely related MHC class I–like genes on chromosome 1 (130). However, the frequency of CD1 protein-specific γδ T cells appears to be very low and in the same order of magnitude as the frequency of CD1 protein-specific αβ T cells (129).

Several human γδ T cell clones recognize a cell surface protein referred to as *T cell target antigen* 1 (TCT.1 or CD48), a member of the Ig superfamily (131–133) encoded by a gene that is located in the same band of chromosome 1 as the CD1 gene cluster (132). CD48 may be an antigen presenting protein or a ligand for a surface protein involved in γδ T cell activation.

We favor the view that γδ T cells recognize peptides and perhaps other small molecules such as carbohydrates in association with nonpolymorphic antigen presenting proteins. So far there are only a few well-documented cases of γδ T cell specificities for such proteins. In contrast αβ T cells specific for allogeneic MHC proteins are readily detectable even in very small αβ T cell population samples. The high alloreactivity of αβ T cells is due to crossreactions of αβ TCRs with a+1 and b or a+1 and b+2, where letters are products of MHC alleles and numbers are presented antigens. Such cross-reactions are not expected for TCRs which recognize non-polymorphic antigen presenting proteins.

Mycobacteria and Heat Shock Proteins

Both murine (Table 6) and human (Table 7) γδ T cells mount strong proliferative responses to killed mycobacteria in the presence of antigen presenting cells. Initially a few γδ T cell lines and clones were obtained from the blood of a BCG immune donor (134), blood or biopsy material from a lepromin skin test of patients with tuberculoid leprosy (135), or synovial fluid of rheumatoid arthritis patients (136). Several of these lines responded to recombinant mycobacterial heat shock proteins. It soon became clear that γδ T cells from healthy donors with negative tuberculin tests and no history of mycobacterial infections also vigorously responded to killed mycobacteria or mycobacterial extracts in the presence of antigen presenting cells and that mycobacterial heat shock proteins were not the major stimulating components (132–139). Every second γδ T cell in the circulating blood of some donors responded to killed mycobacteria, but only a few of the mycobacteria reactive clones also responded to PPD or mycobacterial Hsp65 (137). The major γδ T cell stimulatory components of mycobacteria were found in a small molecular weight fraction (2–10 kd) of extracts, were resistant to proteolytic enzymes, and were shown to

Table 6 Recognition of mycobacteria and/or Hsp by murine $\gamma\delta$ T cells

Origin of $\gamma\delta$ T cells	Specificity	References
B10.A mice were immunized with M.t. in limb; draining lymph node cells were analyzed and restimulated in vitro	*in vivo*: increase in $\gamma\delta$ T cells in draining lymph nodes from 1.5% to 10.4% after immunization *in vitro*: proliferation and IL-2 production in response to M.t.; not blocked by anti-class II MHC antibodies	Janis et al (1989) *Science* 244, 713
BALB/c mice were immunized with aerosol containing M.t. antigen of tuberculin (PPD) or with CFA at the base of the tail	*in vivo*: increase in $\gamma\delta$ T cells in draining lymph nodes *in vitro*: response to heat shocked cells. Specificity for Hsp?	Augustin et al (1989) *Nature* 340, 239 Rajasekar et al (1990) *PNAS* 87, 1767
Thymocytes of B10 newborn mice were fused with BW 5417	Autoreactive $\gamma\delta$ T cell hybridomas (IL-2 production) Additional response to PPD and less well to Hsp65 (BCG) in the presence of spleen cells	O'Brien et al (1989) *Cell* 57, 667
	25 of 26 Vγ1/Vδ6 hybridomas react with PPD Most PPD reactive hybridomas respond to Hsp65 peptide (180–196) No inhibition by anti-class I or II MHC antibodies	Happ et al (1989) *Nature* 342, 696 Born et al (1990) *Science* 249, 67
Spleen cells from adult B10 mice were fused with BW5417	10–20% of hybridomas respond to Hsp60 M.t. and Hsp60 M.t. peptide (180–196); all Vγ1/Vδ6; extensive junctional diversity of both TCR chains All Vγ1/Vδ6 cells are also autoreactive	O'Brien et al (1992) *PNAS* 89: 4348

Table 7 Recognition of mycobacteria and/or Hsp by human γδ T cells

Origin of γδ T cells	Specificity	References
γδ T cell line (GD) was established from PBL of a BCG immune donor after stimulation with PPD and autologous PBMC	Autologous APC+PPD or +rHsp60 (M.t.) Allogeneic APC work less well	Hargewoin et al (1989) *Nature* 340, 309
Four γδ T cell clones were isolated from cultures, containing the synovial fluid T cells of rheumatoid arthritis patients that were repeatedly restimulated with AP-MT	Autologous or allogeneic APC+AP-MT or +purified HSP64 (M. bovis)	Holoshitz et al (1989) *Nature* 339, 226
PBMC (line 1) or skin biopsy cells (line 2) of patients with tuberculoid leprosy were stimulated with M. leprae cell wall antigen and IL2 in the presence of partially HLA matched allogeneic PBMC as APC; long term γδ T cell lines were established after depletion of αβ T cells	Autologous APC+M. leprae PPD (line 1 and 2) or M. leprae cell wall (line 1 and 2) or rHsp65 (BCG) (line 1)* rHsp18 (M. leprae) (line 1)* Tetanus toxin (line 8)* * very weak response	Modlin et al (1989) *Nature* 338, 544
PBL from donors with negative tuberculin test were stimulated with killed M.t. and PBMC as APC in bulk and limiting dilution cultures	Autologous APC+killed M.t. recognized by 1 in 2 to 23 γδ T cells in the blood; only very few of them recognize PPD or Hsp65	Kabelitz et al (1990) *J. Exp. Med.* 171, 667
	All M.t. responsive γδ T cells use Vγ9/Vδ2 TCR	Kabelitz et al (1991) *J. Exp. Med.* 173, 1331
PBL from healthy donors were stimulated with various preparations from mycobacterial lysates and PBMC as APC in bulk and limiting dilution cultures	Autologous APC+protolytic digest (2–10k) of mycobacterial lysates recognized by 1 in 50 to 100 γδ T cells	Pfeffer et al (1990) *Eur. J. Immunol.* 20, 1175
PBL of 22 PPD positive, 2 PPD negative donor and cord blood from 4 neonates were stimulated with killed M.t. and PBMC as APC	Autologous APC+killed M.t. All responding γδ T cells use Vγ9/Vδ2 Vδ2 chains show extensive junctional diversity	Panchamoerty et al (1991) *J. Immunol.* 147, 3360

PBMC and pleural fluid cells of patients with tuberculous pleuritis were stimulated with killed M.t.	Autologous APC+killed M.t. All responding $\gamma\delta$ T cells use Vγ9/Vδ2 Extensive junctional diversity of both TCR chains	Ohmen et al (1991) *J. Immunol.* 147, 3353
Large panel of Vγ9/Vδ2 T cell clones from blood	Autologous APC+killed M.t. Recognized by almost all Vγ9/Vδ2 cells All M.t. specific clones also recognize MOLT-4 cells, some recognize also Listeria and E. coli No class I or II MHC restriction	de Libero et al (1991) *J. Exp. Med.* 173, 1311
T cells from peripheral blood of 5 PPD positive and 3 PPD negative healthy donors were cultured with monocytes either infected with live M.t. or pulsed with killed M.t.	Monocytes infected with M.t. induced expansion of $\gamma\delta$ T cells from all but one donor Monocytes pulsed with killed M.t. induced a lower $\gamma\delta$ T cell response	Havlir et al (1991) *J. Clin. Invest.* 87, 729
$\gamma\delta$ T cells were obtained from peritoneal cavity of mice infected 3 days previously with Listeria monocytogenes	CD4$^-$ CD8$^-$ $\gamma\delta$ T cells produce IFNγ and macrophage chemotactic factor in response to PPD or Hsp65 and irradiated syngeneic spleen cells	Hiromatsu et al (1992) *J. Exp. Med.* 173, 49
$\gamma\delta$ T cells were obtained from the lung of mice primed intranasally with influenza virus and challenged intranasally	$\gamma\delta$ T cells obtained 7 days after challenge express mRNA for IL-2, IL-10, IFNγ, IFNβ, GM-CSF; the frequency of cells producing IL-10 was higher in $\gamma\delta$ than in $\alpha\beta$ T cells	Eichelberger (1991) *J. Immunol.* 147, 2069
$\gamma\delta$ T cell subsets were isolated from intestinal IEL	CD8$^+$ $\gamma\delta$ IEL produce either IFNγ or IL-5 or both; stimulation by anti-$\gamma\delta$ TCR or anti-CD8 mab result in enhanced production of these lymphokines	Taguchi et al (1991) *J. Immunol.* 147, 3736
Splenic and intestinal $\gamma\delta$ T cells were stimulated with anti-CD3 mab	Produce IL-2, IL-3, IFNγ, GM-CSF, IFNα, TGFβ (no IL-4)	Bluestone et al (1991) *Immunol. Rev.* 120, 5

bind to some lectins (138, 139). The chemical nature of this material remains to be elucidated. It also remains to be worked out whether the ligand that is recognized by mycobacteria reactive $\gamma\delta$ T cells is of mycobacterial origin or whether it is an endogenous ligand that is induced by mycobacterial components in antigen presenting host cells. Havlir et al (140) found that $\gamma\delta$ T cells from human blood responded not only to monocytes exposed to dead mycobacteria or PPD but even better to monocytes that were infected with live mycobacteria. This interesting observation needs to be confirmed.

The human $\gamma\delta$ T cells that respond to mycobacteria all use Vγ9 and Vδ2 chains, both of which exhibit considerable junctional diversity (141–144). This suggests that mycobacteria contain or induce a superantigen for human Vγ9/Vδ2 cells.

In mice $\gamma\delta$ T cells accumulated in the draining lymph nodes a few days after immunization with killed mycobacteria in the limb. In vitro these $\gamma\delta$ T cells responded to killed mycobacteria with proliferation and IL–2 production (145). The response was not blocked by antibodies against class II MHC proteins of the host (145). As in humans a high frequency of mycobacteria reactive $\gamma\delta$ T cells was also found in mice. Thus many hybridomas obtained by fusion of BW5147 thymoma cells with thymocytes from newborn mice or spleen cells from adult mice were found to produce IL–2 in response to purified protein derivative (PPD) from mycobacteria (113, 146). Most PPD reactive hybridomas also responded to spleen cells pulsed with recombinant mycobacterial Hsp65 or peptide 180–196 from mycobacterial Hsp65, and less well to the corresponding peptide of murine Hsp63 that is identical in sequence with the corresponding chinese hamster and human Hsp65 peptides (113, 147). The reactivity of these hybridomas with Hsp is not readily demonstrable because of a very high level of spontanous IL–2 production. The response to the exogenous antigens can only be seen when the hybridoma cells are cultured at low density and in the presence of spleen cells. The spontanous production as well as the response to exogenous antigen is inhibitable by antibodies against the $\gamma\delta$ TCR and against the vitronectin receptor but not against MHC class I or class II proteins (113, 148, 149). The endogenous ligand that is responsible for the autoreactivity of the V1 population remains to be defined. It may or may not be a Hsp. So far hybridomas were used to study the specificity of the V1 cells. The autoreactivity of the hybridomas is revealed only in the presence of ligands for the vitronectin receptor (VNR). It is not known whether freshly isolated V1 cells are also autoreactive and whether they express VNRs.

All Hsp responsive $\gamma\delta$ T cell hybridomas were derived from V1 cells and use Vγ1 and Vδ6 TCR chains, both exhibiting extensive junctional diversity

(146, 150, 151). These findings suggest that mycobacterial Hsp peptide 180–196 is a superantigen for V1 cells. The same peptide is recognized by two rat αβ T cell clones one transferring experimental acute encephalitis (EAE) and one protecting against EAE (152).

Superantigens

Staphylococcal enterotoxin A (SEA) is a superantigen for both αβ T cells and γδ T cells (153–155). SEA coated cells are lysed by all human γδ T cells expressing Vγ9 chains. In contrast to αβ T cells the γδ T cells do not proliferate in vitro in response to SEA coated cells. SEA binds on the T cell site to Vγ9 and Vβ and on the antigen presenting site to a non-polymorphic region of MHC class II proteins.

Many of the γδ T cell stimulating cells or agents such as Daudi cells, Molt4 cells, microbial extracts, or heat shock proteins are also suspected to represent or contain superantigens. This suspicion is based on the finding that the TCRs of the responding cells are composed of δ and/or γ chains that use the same variable region gene segments but exhibit extensive junctional diversity. However, the molecular nature of putative superantigens for γδ T cells and their interactions with presenting molecules, as well as with the γδ TCR, remain to be elucidated.

γδ T-Cell Function

γδ T cells have not been noticed by cellular immunologists in innumerable studies of humoral and cell mediated immune responses. It was the discovery of rearranging genes other than Ig and TCRαβ genes rather than the observation of a new function that led to their discovery. Extensive analysis of these cells over the last several years revealed only a few phenotypic differences from αβ T cells.

First, γδ T cells do share many cell surface proteins with αβ T cells such as CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD16, CD25, CD28 or CD45, although the frequency of cells expressing a particular protein and the level of expression vary widely not only between αβ T cells and γδ T cells but also between subsets of γδ T cells and γδ T cells of different species (88, 100–102, 156–162). Besides the γδ TCR, a protein named T19 (also referred to as WC1) is the only unique surface protein of γδ T cells known so far. T19 was discovered by Mackay et al at the surface of γδ T cells from sheep (163). Anti-T19 antibodies stain γδ T cells from other ruminants but not from mice or humans. Recently WC1 cDNA was cloned. It encodes a transmembrane protein of 1436 amino acids with a large extracellular domain that contains 11 repeats that are typical for a family of proteins which includes CD5, CD6, the scavenger receptor, and probably additional WC1 like proteins (164).

Second, like $\alpha\beta$ T cells, $\gamma\delta$ T cells can be stimulated to secrete many lymphokines. The production of different combinations of lymphokines by different $\gamma\delta$ T cell clones has been noticed by several authors (Table 8) (39, 90, 165–176). We do not yet know whether functionally distinct $\gamma\delta$ T cell subsets analogous to the TH1 and TH2 subsets of the $\alpha\beta$ T cell lineage exist.

Third, $\gamma\delta$ T cells resemble $\alpha\beta$ T cells, NK cells and lymphokine activated killer cells (LAK cells) in that they can lyse target cells (177–179) and express the same granule mediators of cytotoxicity such as perforin and serine esterase 1 and 2 (180–182). Cytolytic activity is upregulated by IL–2 as in the other cytolytic cell types mentioned above. Freshly isolated intestinal $\gamma\delta$ T cells from some but not all mouse strains resemble NK cells in that they appear to constitutively express cytolytic activity (180, 183, 184; H. Ishikawa, Y. Li, A. Abeliovich, S. Yamamoto, S. H. E. Kaufmann, S. Tonegawa, submitted).

It is interesting that $\gamma\delta$ T cells isolated from murine skin and human blood share with NK cells the expression of Fc-receptors (murine FcγRα and human FcγRIII also named CD16) which mediate antibody dependent cellular cytotoxicity (ADCC) (185, 186). Because the homogeneous TCR on epidermal $\gamma\delta$ T cells in mice severely restricts antigen recognition, the FcR on these cells may broaden their scope for antigen recognition via aggregated IgG (185).

Like all other lymphocytes, $\gamma\delta$ T cells are under strict control of their antigen receptors. Ligand binding to $\gamma\delta$ TCRs leads to transmission of signal 1 by the CD3 complex (177), which appears to have the same subunit composition as the CD3 complex in $\alpha\beta$ T cells (186a). One or more additional signals mediated by other T cell surface antigens must accompany signal 1 to induce a response such as high affinity IL–2 receptor expression, proliferation, cytolytic activity, or secretion of lymphokines (187–189). The functional role of the various proteins at the $\gamma\delta$ T cell surface remains to be elucidated.

While most mature $\alpha\beta$ T cell express either CD4 or CD8, most $\gamma\delta$ T cells lack both markers (13, 36, 88, 161, 179). However a few $\gamma\delta$ T cells do express either CD8 (36, 88, 89) or CD4 (88, 167, 168, 190, 191), and most intestinal $\gamma\delta$ T cells express CD8α homodimers (46, 192). Analysis of human $\gamma\delta$ T cell clones indicated that CD4 $\gamma\delta$ T cells resemble CD4 $\alpha\beta$ T cells in that activated cells produce lymphokines at high levels but express little or no cytolytic activity (167, 168). The reverse is true for CD8 positive $\alpha\beta$ and $\gamma\delta$ T cells. We conclude that $\gamma\delta$ T cells and $\alpha\beta$ T cells do use the same "tools."

$\gamma\delta$ T cell research has emerged from molecular studies and remains heavily dominated by molecular studies. At the present time functional

studies in vivo are only beginning to address the putative role of γδ T cells in the defense against infections or in various pathological immune responses. The classical method to define the function of a particular cell type is to see what happens if it is eliminated. Recent studies investigate mice (193) and rats (194) depleted of αβ T cells by treatment from birth on with anti-αβ TCR antibodies. The spleens and lymph nodes of these animals contained normal numbers of γδ T cells. A few αβ T cells expressed the αβ TCR at 5 to 10 times lower levels than αβ T cells from untreated mice. αβ T cell–deficient mice were also obtained from embryonal stem cells (ES cells) in which the α or β TCR locus was disrupted by homologous gene recombination (195, 196). These αβ TCR knock out mice have no αβ T cells at all but normal numbers of γδ T cells. Initial results obtained with αβ T cell–depleted mice and rats and with αβ TCR knock out mice are described in the following sections, together with observations that were made in normal mice and in patients with various immunological disorders.

Role of γδ T-Cells in Humoral Immune Responses

γδ T cells have been found to induce Ig secretion in B cell lines (197) and to induce autoantibody production in blood cells of patients with lupus erythematosus (198). A small fraction of human γδ T cells that express the CD4 marker could provide help for antibody responses in vitro (167, 168). The latter study showed that the γδ TCR was not involved in the interaction with B cells (168). The biological significance of this in vitro observation is questionable. Indeed no antibody responses to T cell–dependent antigens were obtained in αβ T cell–depleted mice and rats (193, 194) nor in αβ TCR knock out mice (196; P. Mombaerts, J. Iacomini, S. Tonegawa, unpublished), while antibody responses to type I and type II T cell–independent antigens were the same as in normal mice. These findings suggest that γδ T cells do not normally function as helper cells for B cells. Whether they can suppress B cell responses remains to be seen.

Role of γδ T-Cells in Graft Rejection

The vigorous rejection of grafts from MHC-mismatched donors is due to the high frequency of αβ T cells that recognize allogeneic MHC proteins. As pointed out above γδ T cell populations do not seem to contain many cells that recognize allogeneic MHC proteins or any other polymorphic proteins. No proliferative response was observed in mixed leukocyte cultures containing responder cells from αβ T cell–depleted mice or rats and stimulator cells from MHC disparate strains (193, 194). Even when exogenous IL–2 was added, no response to the allogeneic stimulator cell was seen. Moreover αβ T cell depleted rats failed to reject skin grafts from MHC-disparate donors (194).

Table 8 Lymphokine production by $\gamma\delta$ T cells

Origin of $\gamma\delta$ T cell	Species	Lymphokine	References
Various T cell subpopulations were obtained from blood and stimulated with PPD or PHA	Human	$\gamma\delta$ T cell clones were heterogeneous with regard to lymphokine production, high production of IL-4, IL-5 (clone HD 109), IFNγ (clone IPD2.4) IL-2, IL-4 IFNγ (clone LG.C6) correlation seen only for IL-2 and TBFα Only CD4 $\gamma\delta$ T cell clones produce high levels of IL-2 and GM-CSF	Morita et al (1991) *Eur. J. Immunol.* 21, 2999
Various $\gamma\delta$ T cell clones were established from blood by stimulation with irradiated Jy cells and IL-2 or IL-4	Human	IFNγ and GM-CSF production High by $CD4^+$ $CD28^+$ $CD11b^-$ $\gamma\delta$ T cells Low by $CD8^+$ $CD28^{+/-}$ $CD11b^+$ $\gamma\delta$ T cells	Spits et al (1991) *J. Immunol.* 147, 1180
HLA-A2 specific $\gamma\delta$ T cell clones obtained stimulation of $CD4^-$ $CD8^-$ PBL with irradiated Jy cells and IL-2 or IL-4	Human	$\gamma\delta$ clones product IL-2, IFNγ, GM-CSF Only some produce IL-4	Spits et al (1990) *J. Immunol.* 144, 4150
$\gamma\delta$ T cells were purified from blood of patients with schistosomiasis and carcinoma of the urinary bladder	Human	$\gamma\delta$ T cells produce high levels of BCGF and BCDF but are deficient in IL-2 production	Raziuddin et al (1992) *Eur. J. Immunol.* 22, 309
Freshly isolated blood $\gamma\delta$ T cells were stimulated with anti-CD3 or anti-$\gamma\delta$ TCR mab	Human	IL-2, TNF, IFNγ production	Mingari et al (1987) *Int. J. Cancer* 40, 495 Ferrini et al (1987) *J. Exp. Med.* 166, 277
$\gamma\delta$ T cell clones were stimulated with anti-CD3 or lectin	Human	IL-2, IL-4, IL-5, TNFα GM-CSF, IFNγ production IL-2 and IL-4 production low or undetectable in most clones	Porcelli et al (1991) *Immunol. Rev.* 120, 137

$\gamma\delta$ T cells were obtained from early fetal (8–15 weeks) thymi and postnatal thymi (4–6 months)	Human	Early thymic $\gamma\delta$ T cells produce IL-4, IL-5, GM-CSF, IFNγ Late thymic $\gamma\delta$ T cells produce GM-CSF and IFNγ	Krangel et al (1990) *J. Exp. Med.* 172, 847
$\gamma\delta$ T cell clones were isolated from fetal liver and thymus (14 weeks)	Human	Produce: IL-2, IL-4, IFNγ production (clone 12) IL-4 production (clone T6)	Carding et al (1990) *Eur. J. Immunol.* 20, 1327
$\gamma\delta$ T cell lines PEER and Molt 13	Human	Anti-CD7 mab induce mRNA for TNFα and TNFβ (PEER) and GM-CSF (Molt 13)	Carrel et al (1991) *Eur. J. Immunol.* 21, 1195
$\gamma\delta$ T cells were obtained from peritoneal cavity of mice infected 3 days previously with Listeria monocytogenes	Mouse	$CD4^-$ $CD8^-$ $\gamma\delta$ T cells produce IFNγ and macrophage chemotactic factor in response to PPD or Hsp65 and irradiated syngeneic spleen cells	Hiromatsu et al (1992) *J. Exp. Med.* 173, 49
$\gamma\delta$ T cells were obtained from the lung of mice primed intranasally with influenza virus and challenged intranasally	Mouse	$\gamma\delta$ T cells obtained 7 days after challenge express mRNA for IL-2, IL-10, IFNγ, IFNβ, GM-CSF; the frequency of cells producing IL-10 was higher in $\gamma\delta$ than in $\alpha\beta$ T cells	Eichelberger (1991) *J. Immunol.* 147, 2069
$\gamma\delta$ T cell subsets were isolated from intestinal IEL	Mouse	$CD8^+$ $\gamma\delta$ IEL produce either IFNγ or IL-5 or both; stimulation by anti-$\gamma\delta$ TCR or anti-CD8 mab result in enhanced of these lymphokines	Taguchi et al (1991) *J. Immunol.* 147, 3736
Splenic and intestinal $\gamma\delta$ T cells were stimulated with anti-CD3 mab	Mouse	IL-2, IL-3, IFNγ, GM-CSF, IFNα, TGFβ production; no IL-4 production	Bluestone et al (1991) *Immunol. Rev.* 120, 5

About one third of all lymphocytes that were isolated from endomyocardial biopsies of human heart allografts more than 1 year after transplantation were $\gamma\delta$ T cells, while earlier biopsies contained less $\gamma\delta$ T cells (199). The biopsy derived $\gamma\delta$ T cells were not specific for the donor cells. They were suspected to downregulate immune responses to allogeneic cells.

$\gamma\delta$ T cells have also been studied in patients receiving bone marrow or fetal liver and thymus transplants for treatment of immunodeficiency or neoplastic diseases (200–204). The immune system of most reconstituted patients consists of a complex mixture of host and donor cells and often does not function well for many months after transplantation. Some patients had elevated numbers of circulating $\gamma\delta$ T cells early after the transplantation presumably because $\gamma\delta$ T cells that were present in the graft expanded in the host (200–204). Antidonor reactivity of $\gamma\delta$ T cells has been implicated in the poor function of the immune system in some cases (200). In other cases $\gamma\delta$ T cells from transplanted patients did not show any reactivity to donor or host cells (203).

Role of $\gamma\delta$ T-Cells in Infectious Diseases

In vitro responses of $\gamma\delta$ T cells to microrganism and microbial compounds and related in vivo observations in humans and mice are summarized in Table 9 (bacteria), Table 10 (viruses), and Table 11 (parasites).

$\gamma\delta$ T cells accumulate in the draining lymph nodes of mice infected in the footpad with mycobacteria (145), in the lungs of mice infected intranasally with influenza virus (175, 205), in the peritoneal cavity of mice infected with *Listeria monocygotenes* (174, 206), in the hepatic granulomas of Schistosome infected mice (207), and in the skin lesions of patients with the tuberculous form of leprosy or with cutaneous leishmaniasis (135, 208). Elevated numbers of $\gamma\delta$ T cells have been noticed in the spleens of mice infected with *Trypanosoma cruzi* and *Plasmodium chabaudi* (209), in the blood of patients during the acute and convalescent phases of malaria infections (210, 211). In the acute phase of Epstein Barr virus (EBV) infection, the number of circulating Vδ2 cells was increased (212) while in vitro EBV transformed cells mainly stimulated Vδ1 (213). Elevated numbers of circulating $\gamma\delta$ T cells were in the blood of HIV infected patients with AIDS (214–216). Two of 35 T cell clones obtained from cells in the cerebrospinal fluid of patients with measle virus mediated subacute, sclerosizing panencephalitis were $\gamma\delta$ T cells (217).

Interestingly little evidence suggests that the expansion or accumulation of $\gamma\delta$ T cell clones in the infected tissues is due to the recognition of microbial antigens. A single $\gamma\delta$ T cell clone specific for a viral protein was isolated from the draining lymph node of a mouse infected in the footpad

Table 9 Bacterial infections

Bacteria	Species	Observations	References
Mycobacteria	Mouse	Increase in number of $\gamma\delta$ T cells in draining lymph nodes of mice immunized with M.t. in limb	Janis et al (1989) *Science* 244, 713
	Human	$\gamma\delta$ T cells accumulate granulomatous skin lesions of patients with leprosy (reversal reaction and positive lepromin skin test)	Modlin et al (1989) *Nature* 338, 544
		increased number of $\gamma\delta$ T cells in tuberculous lymphadenitis	Falini et al (1989) *J. Immunol.* 143, 2480
Listeria monocytogenes (L.m.)	Mouse	Increased number of $\gamma\delta$ T cells in peritoneal cavity of mice 3 days after intraperitoneal infection with L.m.	Ohga et al (1990) *Eur. J. Immunol.* 20, 533
		The early appearing $\gamma\delta$ T cells proliferate and secrete IFNγ and macrophage chemotactic factor in response to PPD from M.t. or Hsp65 from *M. bovis* but not to killed Listeria	Hiromatsu et al (1992) *J. Exp. Med.* 175, 49
		Mice depleted of $\alpha\beta$ T cells by mAb treatment show resistance of early stage of infection only	Kaufmann et al pers. commun.
		Treatment with anti-$\gamma\delta$ TCR mAb leads to enhanced L.m. multiplication at an early stage of infection	
Various bacteria	Human	$\gamma\delta$ T cells from blood of healthy donors	Munk et al (1990) *J. Immunol.* 145, 2434
		Proliferation induced by killed mycobacteria, group A streptococci; staphylococcus aureus or Listeria monocytogenes	
		Killing of antigen pulsed target cells reveals recognition of shared and nonshared microbial components	
		A large number of different bacteria, especially gram negative bacteria induce proliferation of $\gamma\delta$ T cells from adult blood or cord blood	Abo et al (1990) *Int. Immunol.* 2, 8
Staphylococcus	Human	All Vγ9 expressing cells lyse target cells pulsed with staphylococcal enterotoxin (SEA)	Rust et al (1990) *Nature* 346, 572

Table 10 Viral infections

Virus	Species	Observations	References
Influenza virus	Mice	Number of $\gamma\delta$ T cells is increased in lung lavage cells 7 to 10 days after intranasal infection	Carding et al (1990) *J. Exp. Med.* 172, 1225
		$\gamma\delta$ T cells may provide protective cover of the lung during the time that tissue repair is proceeding through the secretion of cytokines (IFNγ, IFNβ, GM-CSF) in response to hsp expressing macrophages	Eichelberger et al (1991) *J. Immunol.* 147, 2069
		Alternatively $\gamma\delta$ T cells are passively recruited from blood and play no active part in the disease process	
Herpes simplex virus (HSV-1)	Mice	A $\gamma\delta$ T cell clone was isolated from the draining lymph node of a mouse infected with HSV-1 in the foot pat; biological significance?	Johnson et al (1992) *J. Immunol.* 148, 983
Epstein Barr virus (EBV)	Human	Elevated number of Vγ9/Vδ2 cells in blood in acute phase of EBV infection	de Paoli et al (1990) *J. Infect. Dis.* 161, 1013
		Numbers remain high for 4 weeks	
		EBV transformed B cells selectively stimulate Vδ1 cells in vitro	Hacker et al (1992) *Eur. J. Immunol.* In press
Human immunodeficiency virus (HIV)	Human	Increased numbers of Vδ1 cells in blood of some HIV infected patients; increase is most marked in patients with AIDS	Autran et al (1989) *Clin. Exp. Immunol.* 75, 206
		Vδ1 increase may be due to diminished retention within the thymus that is damaged by HIV infection or to stimulation by activated autologous B cells	de Paoli et al (1991) *Clin. Exp. Immunol.* 83, 187
			Hacker et al (1992) *Eur. J. Immunol.* In press
Measles virus	Human	Subacute sclerosing panencephalitis (SSPE) is due to an immune response against measle virus in the central nervous system; 2 of 35 T cell clones from cerebrospinal fluid of a patient with SSPE were $\gamma\delta$ T cells; no proliferative response to measle virus infected cells was obtained in vitro	Ang et al (1987) *J. Exp. Med.* 165, 1453

Table 11 Parasitic infections

Parasites	Species	Observations	References
Trypanosoma cruzi	Mouse	Selective increase of $\gamma\delta$ T cells in the spleen of C57B1/6 mice infected with *Trypanosoma cruzi*	Minoprio et al (1989) *Immunol. Rev.* 112, 183
Plasmodium chabaudii	Mouse	Increase in number of $\gamma\delta$ T cells in the spleen of BALB/c mice seven days after infection with *Plasmodium chabaudii*	Minoprio et al (1989) *Immunol. Rev.* 112, 183
Plasmodium falciparum	Human	Increase in number of $\gamma\delta$ T cells in blood during acute and convalescent phases of malaria infections	Roussilhon et al (1990) *J. Inf. Dis.* 162, 283 Ho et al (1990) *Immunol. Letters* 25, 139
	Mouse	Outgrowth of Vγ9 cells from peripheral blood stimulated in vitro with merozoites, schizont lysate or whole parasitized red blood cells	Goerlich et al (1991) *Eur. J. Immunol.* 21, 2613 Behr & Dubois (1992) *Int. Immunol.* 4, 361 Goddier et al (1992) *Int. Immunol.* 4, 33
Cutaneous Leishmaniasis	Human	Accumulation of $\gamma\delta$ T cells in granulomatous skin lesions	Modlin et al (1989) *Nature* 339, 544
Schistosomiasis	Human	Increased number of $CD4^+$ $CD8^-$ $\gamma\delta$ T cells in the blood	Raziuddin et al (1992) *Eur. J. Immunol.* 22, 309
	Mice	In hepatic granulomas of schistosome infected mice activated $\gamma\delta$ T cells are present and express high levels of IgA and IgM FcR and low levels of IgG FcR; ADCC by $\gamma\delta$ T cells?	Sandor et al (1992) *J. Immunol.* 148, 2363

with *Herpes simplex* virus (218). Recently Flynn found $\gamma\delta$ T cells specific for various trypanosome proteins in the blood of cattle following infection with *trypanosoma congolense*. Interestingly such responses were observed in West African N'Dama cattle which recover from trypanosoma infections but not in Boran cattle which succumbed to the infection (N. Flynn, personal communication).

We are not aware of any other case in which $\gamma\delta$ T cells were unequivocally shown to recognize and respond to defined microbial antigens in infected humans or animals. The $\gamma\delta$ T cells in the lungs of mice infected with influenza virus appear to recognize host cell components rather than viral antigens (175). Similarly the $\gamma\delta$ T cells that accumulate in the peritoneal cavity of mice 3 days after infection with *Listeria monocygotenes* proliferate and secrete IFNγ and macrophage chemotactic factor in response to putative mycobacterial superantigens but not to listerial antigens (174). Mice depleted of $\alpha\beta$ T cells by mab treatment show resistance to the infection by *Listeria monocygotenes* within the first few days after infection but cannot control the infection later (S. H. Kaufmann, P. Mombaerts, unpublished observation). This finding is consistent with the well-established protective role of *Listeria monozygotenes*-specific $\alpha\beta$ T cells. $\gamma\delta$ T cells appear to have a crucial function early after infection at a time when there is no protection by specific $\alpha\beta$ T cells. An exaggerated multiplication of Listeria microorganisms is seen in normal mice and $\alpha\beta$ TCR knock out mice that were treated with anti-$\gamma\delta$ TCR antibodies (S. H. Kaufmann, P. Mombaerts, unpublished observation). These findings are the first indication for complementary functions of $\alpha\beta$ and $\gamma\delta$ T cells. The protection by $\gamma\delta$ T cells is not Listeria specific. It is fast but incomplete. For survival $\alpha\beta$ T cells must recognize and respond to listerial antigens and mount an immune response that eliminates the pathogen. Recently similar observations were made in a mouse model of malaria (R. Nussenzweig, personal communication).

Immune Surveillance Against Cancer by $\gamma\delta$ T-Cells

$\gamma\delta$ T cells have been suspected to have a surveillance function against tumors. The following observations (summarized in Table 12) are cited to support this idea:

First, IL–2 activated $\gamma\delta$ T cells can kill many different tumor cells. The molecular basis of the distinction between normal cells and tumor cells by the lymphokine activated $\gamma\delta$ T cell cells is not known.

Second, a subset of human $\gamma\delta$ T cells appears to recognize a superantigen on the surface of some Burkitt lymphoma cells (219–223). Another subset recognizes Epstein Barr virus transformed B cells (213).

Third, some $\gamma\delta$ T cells that were isolated from patients with Burkitt

lymphomas or acute lymphoblastic leukemias (ALL) in complete remission were shown specifically to recognize autologous tumor cells (224).

Fourth, γδ T cell lines could be established from populations of tumor infiltrating lymphocytes (TIL) (225–227). The γδ TIL isolated from lung tumors lysed autologous tumor cells, and this lysis was inhibited by anti-class I MHC antibodies (227). No evidence for specific lytic activity was obtained with γδ TIL that were isolated from Wilms tumors, melanomas, and sarcomas (225). These observations are interesting but far from being convincing evidence for a role of γδ T cells in the defense against tumors.

Pathological Immune Responses by γδ T-Cell

γδ T cells have been suspected to contribute to pathological immune responses in several different diseases (Table 13). The synovial fluid from patients with rheumatoid arthritis was found by many investigators to contain γδ T cells of the V1 subset that represents a minor proportion of circulating γδ T cells in most healthy individuals (136, 228–238). Vδ1 cells also accumulate in the intestinal lesions of patients with coeliac disease (239–243) and were found increased in the blood of a patient with type I autoimmune polyglandular syndrome that was associated with aplastic anemia (244). Vδ2 cells were found in 10 of 10 brain autopsies from multiple sclerosis patients (238) and the number of Vδ2 cells was elevated in the blood of a patient with atopic dermatitis (245).

The analysis of junctional regions of TCR chains from γδ T cells of patients with rheumatoid arthritis (232, 236–238), multiple sclerosis (242) or coeliac disease (243) did not reveal any extensive expansion of particular γδ T cell clones. However large γδ T cell clones were found in the blood and the bronchoalveolar lavage of some patients with pulmonary sarcoidosis (246, 247).

These reports are interesting but do not document a pathogenic or protective role of γδ T cells.

γδ T-Cells in Immunodeficiency Diseases

Brenner et al were the first to identify peripheral blood T cells which were CD3 positive but αβ TCR negative (8). While the initial attempts to expand these cells from the blood of normal donors failed, since the cultures were quickly overgrown by αβ T cells, these investigators succeeded in obtaining two $CD3^+$, αβ TCR^- lines from one patient with bare lymphocyte syndrome (IDP1) and one patient with ectodermal dysplasia syndrome (IDP2) respectively. These were the first cells from which CD3 cross-linked γδ TCRs were precipitated (8). The two TCR chains of both lines were non-disulphide linked. Later several additional lines and clones expressing either non-disulfide linked or disulfide linked γδ TCRs were obtained from

Table 12 Recognition of neoplastic cells by $\gamma\delta$ T cells

Cancer	Species	Observations	References
B cell lymphoma	Mouse	$\gamma\delta$ T cells proliferate in response to CD5 positive B cell lymphomas and induce Ig secretion	Sperling & Wortis (1989) *Int. Immunol.* 1, 434
Burkitt's lymphoma and EBV transformed B-LCL	Human	$\gamma\delta$ T cells from children with Burkitt lymphoma lyse autologous tumor cells; the $\gamma\delta$ T cell tumor cell interaction involves the $\gamma\delta$ TCR and surface Ig of the tumor	Wright et al (1989) *J. Exp. Med.* 169, 1557
		Daudi Burkitt lymphoma cells induce selective growth of Vγ9/Vδ2 T cells; EBV transformed B-LCL induce growth of Vγ1 T cells	Hacker et al (1992) *EJI.* Submitted
		Many of 356 $\gamma\delta$ T cell clones lyse Daudi but not Raji Burkitt lymphoma cells while NK cell lyse both targets	Fisch et al (1990) *J. Exp. Med.* 171, 1567
		All $\gamma\delta$ T cell clones which lyse Daudi cells express Vγ9 Vδ2 TCR; the Vδ2 chains show extensive junctional diversity	Sturm et al (1990) *J. Immunol.* 145, 3202
		Freshly isolated Vγ9/Vδ2 T cells from blood proliferate in response to Daudi cells	
		Lysis of Daudi cells by $\gamma\delta$ T cells is inhibited by a rabbit antiserum against mammalian Hsp50	Fisch et al (1990) *Science* 250, 1269

		Most $\gamma\delta$ T cells expressing Vγ9/Vδ1 TCR also recognize Daudi cells in cytotoxicity and proliferative assays	Fisch et al (1990) *J. Immunol.* 148, 2315
Lung carcinoma	Human	The majority of freshly isolated tumor infiltrating lymphocytes from 2 patients were $\gamma\delta$ T cells; 100% and 40% respectively were V$\delta1^+$; the $\gamma\delta$ T cells lysed autologous tumor cells; lysis was inhibited by anti-class I MHC antibodies	Zocchi et al (1990) *Eur. J. Immunol.* 20, 2685
Wilms tumor Sarcoma Melanoma	Human	$\gamma\delta$ T cell lines obtained from tumor infiltrating lymphocytes by stimulation with autologous tumor cells and IL-2; no evidence for tumor specific lysis	Nanno et al (1992) *Eur. J. Immunol.* 22, 679
Localized pagetoid reticulosis (Woringer-Kolopp disease)	Human	$\gamma\delta$ T cells are abundant in epidermal infiltrate	Alaibac (1992) *Int. J. Dermatol.* 31, 157
Langerhans cell histiocytosis	Human	$\gamma\delta$ T cells are increased in number in the dermal and epidermal infiltrate in close association with Langerhans cells	Alaibac (1992) *Int. J. Dermatol.* 31, 157
Acute lymphoblastic leukemia (ALL)	Human	84 $\gamma\delta$ T cell clones were isolated from blood of 3 ALL patients after complete remission was achieved; all clones express cytolytic activity but only 10 clones lysed autologous leukemia cells	Bensussan et al (1989) *Blood* 73, 2077

Table 13 Pathological immune responses

Disease	Species	Observations	References
Rheumatoid arthritis (RA)	Human	10 of 202 T cell clones from synovial fluid of 4 juvenile arthritis patients are probably $\gamma\delta$ T cells	DeMaria et al (1987) *Eur. J. Immunol.* 17, 1815
		$\gamma\delta$ T cells are enriched in synovial fluid as compared to blood in some patients with RA	Brennan et al (1988) *J. Autoimmunity* 1, 319
		Coordinate expansion of $\gamma\delta$ T cells and $CD5^+$ B cells in blood of patients with RA and primary Sjogren's syndrome	Brennan et al (1989) *Clin. Exp. Immunol.* 77, 175
		4 $\gamma\delta$ T cell clones isolated from synovial fluid of a patient with RA respond to acetone precipitable fraction of M.t.	Holoshitz et al (1989) *Nature* 339, 226
		$\gamma\delta$ T cells in synovial fluid of RA patients preferentially use Vδ1	Sioud et al (1990) *Scand. J. Immunol.* 31, 415
		Vδ1 chains have diverse junctional sequences	Sioud et al (1991) *Eur. J. Immunol.* 21, 239
		$V\delta1^+$, $CD69^+$, $CD25^+$ $\gamma\delta$ T cells in synovial compartment of 6 juvenile RA patients	Kjeldsen-Kragh et al (1990) *Scand. J. Immunol.* 32, 651
		Lower numbers of $\gamma\delta$ T cells in blood of RA patients	Smith et al (1990) *Scand. J. Immunol.* 32, 585
		No consistent increase of $\gamma\delta$ T cells in synovial fluid or tissues of RA patients	Pope et al (1991) *Cell. Immunol.* 137, 127
		Increased number of $\gamma\delta$ T cells in synovial fluid of RA patients show extensive junctional diversity except in one patient	Keystone et al (1991) *Clin. Exp. Immunol.* 84, 78
		Vδ1 chains of $\gamma\delta$ T cells in synovial fluid of RA patients show extensive junctional diversity except in one patient	Bucht et al (1992) *Eur. J. Immunol.* 22, 567
Multiple sclerosis (MS)	Human	Identification of $\gamma\delta$ T cells in MS brain lesions by immunohistochemical techniques	Selmaj et al (1991) *PNAS* 88, 6425
		Evidence for clonal expansion of $\gamma\delta$ T cells in MS brain lesions	Wucherpfennig et al (1992) *PNAS*. In press
		γ and δ transcripts were found in 12 of 12 MS brains but only in 1 of 10 control brains junctional diversity of Vδ2 chains was limited	Huas et al (1992). Submitted

Coeliac disease	Human	Elevated number of $\gamma\delta$ T cells ($V\delta_1+$) in intestinal epithelia of patients with coeliac disease	Spencer et al (1989) *Eur. J. Immunol.* 19, 1335 Viney et al (1990) *Gut* 31, 841 Trejdosiewicz et al (1991) *Clin. Exp. Immunol.* 84, 440
		No evidence for oligoclonal $\gamma\delta$ T cell expansion	Rust et al (1992) *Scand. J. Immunol.* 35, 459 DeLibero et al 1992. Submitted
Polymyositis	Human	$\gamma\delta$ T cell infiltrates in nonnecrotic muscle fibres of one patient	Hohlfeld et al (1991) *New Eng. J. Med.* 324, 877
Kikuchi's lymphadenitis	Human	Hyperimmune response to unknown antigen massive $\gamma\delta$ T cell infiltrates	Falini et al (1989) *J. Immunol.* 143, 2480
Lupus nephritis	Human	7 of 59 autoantibody inducing TH lines obtained from blood were $\gamma\delta$ T cells	Rajagopalan et al (1990) *PNAS* 87, 7020
Discoid chronic lupus erythematosus	Human	$\gamma\delta$ T cells accumulate in dermis and basal keratinocyte layer of epidermis, frequently surrounding damaged keratinocytes	Platzer et al (1990) 20th Annual Meetg. Eur. Society of Dermatological Research
Type I Autoimmune polyglandular syndrome	Human	Increase in $V\delta1^+$ cells in blood associated with aplasic anemia	Hara et al (1990) *Blood* 75, 941
Atopic dermatitis	Human	$V\delta2^+$ cells expanded in blood of one patient	de Paoli et al (1990) *Immunol. Letters* 23, 195
Pulmonary sarcoidosis	Human	7 of 20 patients have elevated $\gamma\delta$ T cells in blood and bronchoalveolar lavage from lower respiratory tract	Balbi et al (1990) *J. Clin. Invest.* 85, 1353
		Expansion of a single clone (homogeneous junctional sequences) in blood and lung of one patient	Tamura et al (1990) *J. Exp. Med.* 172, 169

patients with different primary immunodeficiency diseases such as partial DiGeorge syndrome, common variable immunodeficiency (248), or Wiskott Aldrich syndrome (249). The studies published so far on $\gamma\delta$ T cells from patients with primary immunodeficiency syndroms are rather fragmentary, however.

Elevated numbers of $\gamma\delta$ T cell were also found in the blood of patients with Down's syndrome who are more prone to autoimmune diaseases, have a greatly increased susceptibility to viral and bacterial infections, and a 10- to 20-fold higher incidence of childhood leukemia (250). At least 5 of 10 patients with ataxia-teleangiectasia had an increased ratio of $\gamma\delta$ T cells to $\alpha\beta$ T cells. This finding is thought to reflect both a recombinational defect that interferes with Ig and TCR gene rearrangements and an inability to repair damage to the DNA (251).

CONCLUDING REMARKS

We do believe that $\gamma\delta$ T cells are as important for defense against microbes as B cells and $\alpha\beta$ T cells. There are many reasons why their function has not yet been recognized. One is certainly the preconception about T cells that is derived from our knowledge of $\alpha\beta$ T cells. Most investigators searching for $\gamma\delta$ T cell functions designed their experiments as if $\gamma\delta$ T cells were just other $\alpha\beta$ T cells. It was assumed that $\gamma\delta$ T cells recognize peptides presented by MHC or MHC-like proteins on the surface of macrophages or dendritic cells. Indeed, spleen cells appear to be appropriate antigen presenting cells, MHC and MHC like proteins are recognized at least by some $\gamma\delta$ T cells, and $\gamma\delta$ T cells responded to antigen recognition with proliferation, lymphokine production, expression of cytolytic activity or anergy—just like $\alpha\beta$ T cells. We do suspect that the observed responses were either mediated by rare $\gamma\delta$ T cells that are not representative for entire $\gamma\delta$ T cell subsets, or the responses were directed to superantigens that activate large fractions of cells irrespective of the junctional diversity of their receptors. The great diversity of $\gamma\delta$ T cells must be crucial for their function. Physiologically relevant responses of $\gamma\delta$ T cells may be directed to peptides with postranslational modifications that occur only in microbes or even to nonpeptidic antigens such as carbohydrates that are presented by novel antigen presenting proteins which coevolved with Vγ and Vδ gene segments. We suspect that there is a diverse set of antigen presenting cells that utilize different antigen presenting proteins in different tissues. This possibility has not been explored extensively. The analysis of these cells and of their mode of antigen presentation may be the key to the understanding of the unique functions of $\gamma\delta$ T cells.

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T CELL RESPONSES TO PRE-ERYTHROCYTIC STAGES OF MALARIA: Role in Protection and Vaccine Development Against Pre-Erythrocytic Stages

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KEY WORDS: malaria vaccine, cytotoxic T cells, circumsporozoite protein, polymorphism, sporozoite immunization, exoerythrocytic stages

Abstract

Malaria remains a leading cause of human morbidity and mortality due to the inability of insecticides and chemotherapy/chemoprophylaxis to eliminate the vectors or disease caused by this protozoan parasite. In an effort to develop new methods of control, vaccines targeted to the various stages of the complex life cycle of Plasmodium have been developed. This review describes recent advances in the elucidation of cell-mediated immune mechanisms directed against sporozoites and liver stages of malaria parasites, their role in protection, and their relation to vaccine development. Recent data on the molecular basis of sporozoite-liver cell interaction are presented, and these may provide new approaches for chemoprophylaxis and immunoprophylaxis. We describe the role of the circumsporozoite protein, the major sporozoite surface antigen, in sporozoite movement and as a target of humoral immunity. The recognition of the circumsporozoite protein by human T cells is reviewed with emphasis on cytotoxic T cells and immune resistance against the exo-erythrocytic stage of the parasite. Earlier concepts regarding the polymorphisms of the

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circumsporozoite protein, the immunological relevance of this polymorphism, and predictions regarding vaccine development are reevaluated on the basis of recent data from different malaria endemic areas. Non-CS sporozoite antigens and liver stage antigens are discussed as potential targets for immune intervention. Recent experimental approaches such as multiple antigen peptides, recombinant live vectors, and new more potent adjuvants are considered for the development of more effective malaria vaccine formulations.

INTRODUCTION

Malaria, which affects approximately 300 million individuals per year, is one of the most prevalent severe infectious diseases in tropical areas throughout the world. Attempts to control the spread of malaria, by combatting the mosquito vectors, have failed in most developing countries. While some of the vectors have become resistant to insecticides that were originally very efficient, other vectors are not strictly domiciliary and are therefore difficult to eliminate. The frequent lack of medical assistance and the unavailability of adequate medication for the local populations compound the difficulties of control.

In the last 10 years the situation has been further aggravated by the very rapid spread of drug resistant *P. falciparum*, the parasite that most frequently causes severe, often life threatening, complications.

Vaccination has traditionally been the least costly, most effective measure of disease control. The efforts of several groups of investigators to develop a malaria vaccine have been quite intensive, particularly in the last 10 years, as monoclonal antibodies, peptide synthesis, and recombinant DNA methodologies have become available. These methodologies have been essential for progress in this area, in view of the impossibility of growing in vitro adequate amounts of the various developmental stages of the four different species of parasites which cause malaria in humans.

While an effective malaria vaccine is not yet available, considerable insight has been gained into the complex, multiple immune mechanisms induced by the different developmental stages of these parasites, their complex antigenic make-up, and their variability.

Life Cycle and Focus of Review

The successive developmental stages of plasmodia provide a number of potential targets for immune intervention. The life cycle of the malaria parasite occurs in two hosts, the *Anopheles* mosquito and a mammalian host such as man (Figure 1). Sporozoites, found in the mosquito's salivary glands, are injected by mosquito bite and rapidly enter liver cells. There

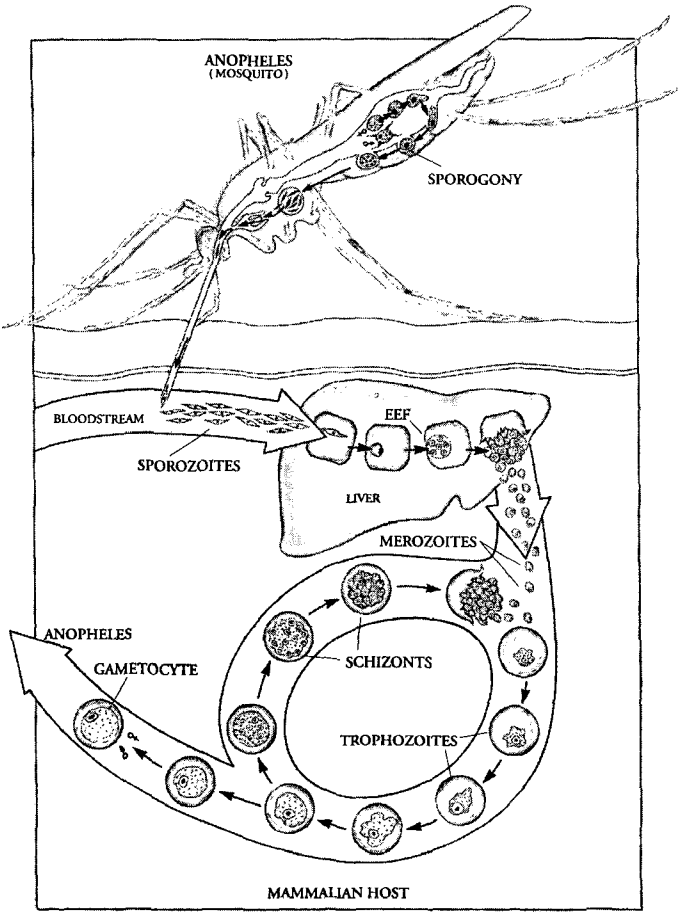


Figure 1 The life cycle of *Plasmodium* in the *Anopheles* mosquito vector and the mammalian host.

they develop into exo-erythrocytic forms (EEF) or liver schizonts, which after a few days mature into thousands of merozoites that enter red blood cells following rupture of the infected hepatocytes.

The growth of the erythrocytic stages is followed by synchronous rupture of the infected red blood cells, causing the typical symptoms of malaria. The *Plasmodium* life cycle is completed when blood stage gametocytes, taken up by the *Anopheles* vector, undergo fertilization and sporogony resulting in the production of infective sporozoites within the mosquito salivary glands. The basis of vaccines aimed at blood stages of

the parasite and transmission blocking immunity has recently been reviewed (1, 2).

This review focuses exclusively on pre-erythrocytic stages, i.e. sporozoites and intrahepatocytic forms, their antigens and interaction with host cells, and the respective immune responses. Earlier findings on the characteristics of sporozoite-induced protective immunity, which provided the basis for much of the subsequent research on sporozoite antigens and vaccine development, were reviewed in 1980 by Cochrane et al (3). The key findings on the structure of the circumsporozoite (CS) protein and the humoral and cellular responses to this antigen, as well as the inhibitory effect of certain cytokines on the intrahepatocytic stages, are summarized in recent reviews (4–6).

Much of the emphasis of our current review is on novel aspects of this host-parasite interaction, uncovered in the last few years. These include recent data on the molecular basis and site of sporozoite-liver cell interaction and the role of the CS protein in sporozoite movement and infectivity. We also summarize what has been learned about the recognition of the CS protein by T cells, in particular cytotoxic T cells, which have recently been shown to inhibit the development of intrahepatocytic stages of rodent malaria and to confer resistance on mice against sporozoite challenge.

While many of the new findings relate to the CS protein, we have also included recent data on additional sporozoite and liver stage antigens, their immunogenicity, and their possible functional role.

In light of currently available data, we evaluate some earlier findings on the variability of certain domains of the CS protein. Particular emphasis will be given to the earlier interpretation of the functional role of these amino acid substitutions, and to some of the predictions concerning vaccine development made at that time.

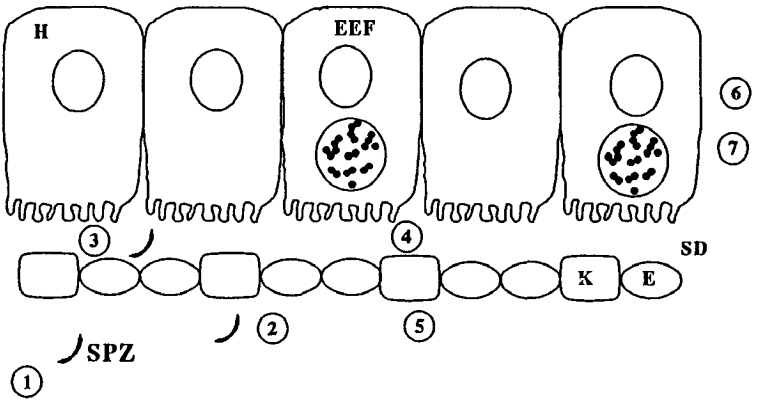
Finally we briefly discuss the approaches being used to incorporate the current findings into the design of more effective synthetic and recombinant vaccine candidates. These include multiple antigen peptides (MAPs), various live vectors, new and more potent adjuvants, and novel vaccine formulations.

Sporozoite-Host Cell Interaction

MOVEMENT OF SPOROZOITES; ROLE IN INFECTIVITY *Plasmodium* sporozoites have been shown to exhibit a characteristic gliding motility (7). This motility is associated with the secretion of CS protein from the apical end of the sporozoite, and the posterior translocation of the CS protein along the sporozoite's pellicle to the posterior end (8). There the CS protein is

released in the form of vesicles, making up a trail left behind by the sporozoite (9, 10). Evidence has been presented that motility of sporozoites is necessary for invasion of host cells (11).

It has been proposed that antibodies against the repeat region of the CS protein function by blocking sporozoite motility (Figure 2). Cross-linking of CS protein molecules by anti-CS antibodies blocks the posterior trans-



PRE-ERYTHROCYTIC IMMUNE EFFECTOR MECHANISMS

HUMORAL	CELLULAR
1. INHIBIT MOTILITY	4. γ IFN BY $CD4^+$ T CELLS
2. INCREASE PHAGOCYTOSIS	5. CYTOKINES IL-1, IL-6
3. BLOCK INVASION	6. CYTOTOXIC T CELLS
	7. γ IFN PRODUCED BY CTL

H-HEPATOCYTE; EEF-EXOERYTHROCYTIC FORM; SD- SPACE OF DISSE

K- KUPFFER CELL; E- ENDOTHELIAL CELL; SPZ- SPOROZOITE

Figure 2 Diagram of the extracellular (sporozoite) and intracellular (EEF) targets of humoral and cell mediated immunity directed at the pre-erythrocytic stages of the malaria parasite.

location of the CS protein (11), thereby inhibiting sporozoite motility and invasion of host cells. Furthermore, sporozoites so immobilized will be phagocytosed more frequently by host macrophages (12).

Role of the CS protein as ligand in sporozoite-hepatocyte interaction A considerable amount of information has been obtained on the structure of the CS protein, as well as on its biosynthesis and evolution. Much has also been learned about the interaction of various domains of the CS protein with the host's immune system. However, the question of what role the CS protein plays in the parasite's life cycle remained, until very recently, unanswered. One clue was provided by the observation that a conserved region in the C terminus of the CS protein, region II, has a high degree of amino acid sequence homology to portions of a cell adhesion molecule, thrombospondin, and to other sulfatide-binding proteins (13). This suggested that the sporozoites might also use region II as a ligand for a sulfated cellular receptor in the liver. Experiments from two groups of investigators then showed that the CS protein from various malaria parasites bound to sulfatides (14, 15) and to cholesterol-sulfate (14). Truncated recombinant CS molecules lacking region II were inactive (14). The binding activity was dependent on disulfide-bond formation between two cysteines in region II (14). Mutant cells, deficient in the biosynthesis of heparan sulfate, did not bind the recombinant protein (15). Various sulfated molecules such as heparan sulfate, dextran sulfate, fucoidin, and heparin inhibited the in vitro invasion of hepatocytes by sporozoites (14, 15). Sporozoite invasion in vivo was also inhibited if the parasites were preincubated with dextran sulfate and fucoidin (15).

Recent findings shed light on these intriguing observations and, in addition, provide a rational explanation for the remarkable target-cell specificity of malaria sporozoites, which rapidly find their way into the hosts' hepatocytes, following their injection by the mosquitoes. It was recently demonstrated that the CS protein binds specifically to areas of the hepatocyte plasma membrane that are exposed to the blood stream (16): the microvilli protruding in the Disse space and the lateral sides of the plasma membrane up to the tight junctions which seal the bile caniculi (Figure 2). The CS protein did not bind to endothelial cells or Kupffer cells in the liver, nor to cells of several other organs, including the spleen and lung. The binding of the CS protein to hepatocytes was specifically inhibited by synthetic peptides containing a redefined (16) region II motif EW—C-VTCG-G—-R-R(K) (Figure 3). Although the precise boundary of the liver hepatocyte ligand has not been delimited, the cysteines are important because activity is lost if they are substituted by alanines. The active peptides, as well as polyclonal antibodies against them, inhibited

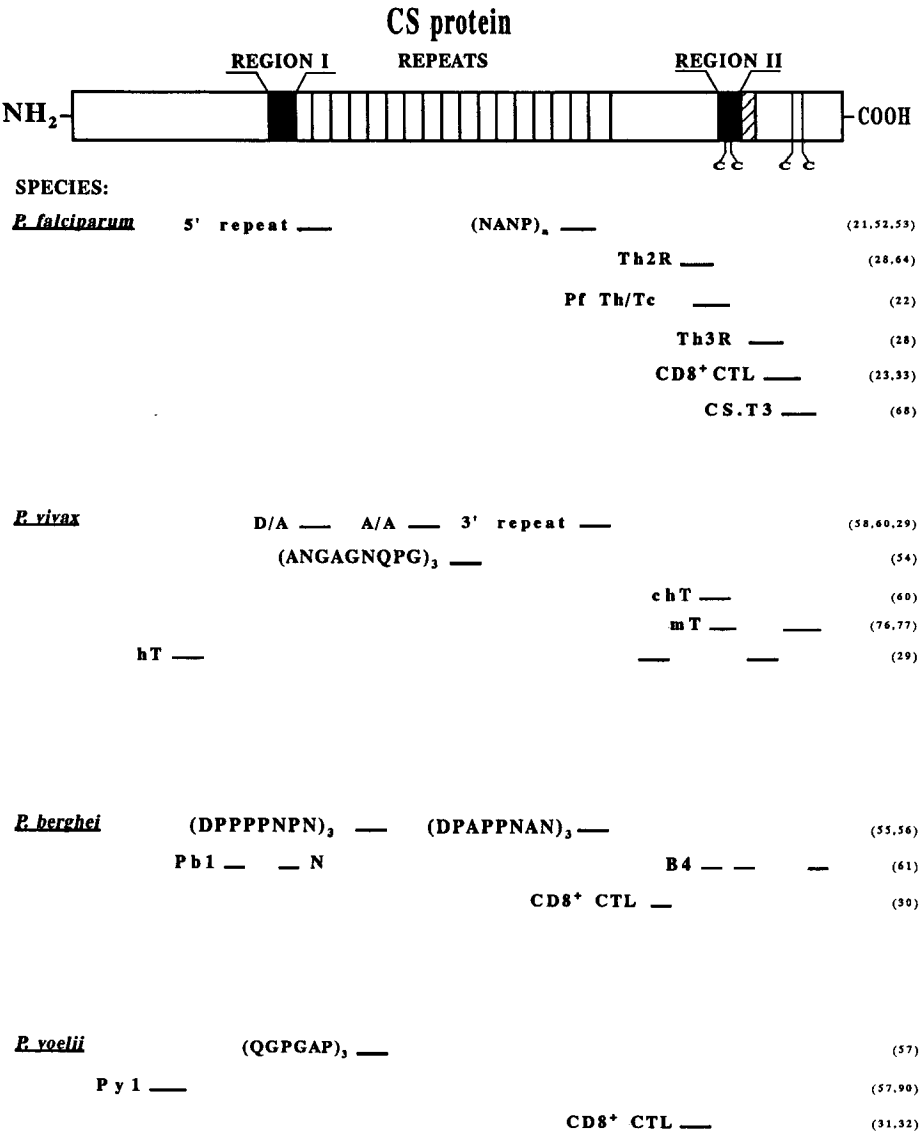


Figure 3 Schematic representation of the CS protein and the T cell epitopes located within repeat (central blocks) and nonrepeat sequences. The conserved region I and II are shown as dark blocks, and the additional amino acids contained in the redefined region II, as described in Ref. 16, are indicated by hatched block. The T cell epitopes are indicated by a solid line marking their approximate location in the CS protein of each species of malaria. Reference(s) for each epitope are in parentheses. ChT, mT and hT refer to epitopes recognized by chimpanzee, murine, or human T cells, respectively.

sporozoite invasion of hepatocytes in vitro (16). The identification of the hepatocyte receptor and further characterization of the ligand could provide new approaches for chemoprophylaxis and immunoprophylaxis of malaria infection.

Sporozoite-induced immunity Successful vaccination of humans against malaria was first accomplished in the 1970s using x-irradiated *Plasmodium*-infected mosquitoes to deliver attenuated, highly immunogenic sporozoites (reviewed in 17, 18). This was based on a large body of experimental work using rodents and monkeys immunized with irradiated sporozoites, which under these conditions develop solid protection against sporozoite challenge (3). Of the 11 human volunteers who at that time were immunized by multiple exposures to irradiated *P. falciparum*-infected mosquitoes, 5 were fully protected against challenge by the bites of mosquitoes containing infective sporozoites of various strains of *P. falciparum*. Similar results were obtained with other volunteers, immunized with irradiated mosquitoes infected with *P. vivax*, another highly prevalent human malaria. At that time, the only available assay to monitor the immune response of these vaccinees was the circumsporozoite precipitation (CSP) reaction, a highly specific but only moderately sensitive assay, which nevertheless appeared to correlate well with protection.

Two groups of investigators recently repeated these vaccine trials by immunizing a small number of volunteers, by the bite of *P. falciparum*-infected, irradiated mosquitoes (19, 20). Their purpose was (*a*) to determine the reproducibility of sporozoite-induced protection and (*b*) to characterize the antibody and cellular responses in protected individuals. Six of nine volunteers who were immunized repeatedly over a period of several months were totally protected when challenged by the bites of five *P. falciparum*-infected mosquitoes. Two of these volunteers were rechallenged with a heterologous strain of *P. falciparum* and shown to be protected.

All the vaccinees developed very high anti-sporozoite antibody titers, measured by immunofluorescence and ELISA. Sera obtained on the day of challenge inhibited sporozoite invasion of hepatoma cells in vitro.

Proliferative responses to recombinant CS protein were detected in peripheral blood lymphocytes (PBL) obtained from the sporozoite-immunized volunteers. The kinetics of the CS-specific cell-mediated immune responses varied among the volunteers and did not correlate with the anti-sporozoite antibody response (19).

$CD4^+$ T cell lines and clones obtained from the peripheral lymphocytes of these vaccinees led to the identification of two T cell epitopes in the *P. falciparum* CS protein, a novel T helper cell epitope located in the repeat domain, and a Th/Tc epitope located in the C-terminus (21, 22).

$CD8^+$ cytotoxic CS-specific T cells were also detected in the peripheral blood of three out of four sporozoite-immunized volunteers (23). These $CD8^+$ CTLs could be stimulated by a peptide representing a variable domain of the C-terminal region of the CS protein. The ability to detect the $CD8^+$ CTL in multiple blood samples was sporadic, probably indicating that these CTLs were present in low numbers. No clear correlation could be established between protection and the presence of CS specific $CD8^+$ cytotoxic T cells in the peripheral blood of the vaccinees.

PROTECTIVE ANTIGENS IN SPOROZOITE-INDUCED IMMUNITY

Circumsporozoite Protein

STRUCTURE/FUNCTION AND POLYMORPHISM Since we last reviewed the subject (5), the CS gene of numerous, geographically distinct isolates of *P. falciparum* and *P. vivax*, as well as of a number of additional Plasmodial species, have been cloned and sequenced. These include the CS genes of several simian and primate malarias such as *P. brasilianum*, *P. simium*, and *P. reichenowi*.

A survey of the sequences of the corresponding proteins corroborates the notion that all CS proteins have a similar basic structure, shown diagrammatically in Figure 3. They are typical membrane proteins, and all have a central region of repeats, which occupies more than one third of the molecule. This central region consists of a series of tandem repeats of small numbers of amino acids, which differ in composition and length in every plasmodial species. These repeats also differ in distinct isolates of the *P. vivax-P. cynomolgi* complex (24, 25). The immunodominant B cell epitope of all known CS proteins is contained in these repeats, and most of the monoclonal anti-sporozoite antibodies generated to date bind to the repeats and neutralize sporozoite infectivity.

Immediately preceding the 5′ end of the repeat region in all CS proteins is a conserved sequence of five amino acids (KLKQP), which has been designated region I (26). In the C-terminal region, all CS proteins have two conserved pairs of cysteines. When comparisons were made between the first two CS proteins that were cloned, the amino acid sequence surrounding the first pair of cysteines was shown to be conserved and was designated region II (26). Region II has recently been redefined on the basis of comparisons of several other cloned CS proteins (16) (Figure 3). This region functions as a ligand mediating the interaction of sporozoites with a hepatocyte receptor (14, 16). Region I may also contribute, in a not yet clearly defined manner, to sporozoite-liver cell interaction (27).

Properties of the CS proteins of rodent malarias The analysis of the

structure/function relationship of the CS protein, particularly with regard to the immune recognition of defined domains of this antigen has been conducted in great detail for the rodent malarias, *P. berghei* and *P. yoelii*. Recombinant CS proteins of both *P. berghei* and *P. yoelii* are recognized by mice of many different H–2 haplotypes. These CS proteins contain numerous T helper epitopes that are frequently recognized by inbred mice of different strains.

These findings were corroborated by field studies on human malaria which documented the presence and frequent recognition of multiple T cell epitopes in the CS protein of *P. falciparum* (28), and more recently also of *P. vivax* (29).

The induction of CS-specific cytotoxic T cells by sporozoite immunization was first demonstrated in two rodent malaria systems (*P. berghei* and *P. yoelii*) (30, 31). $CD8^+$ CTL were used to map the respective epitope in each CS protein. The functional role of these CTLs was demonstrated by the passive transfer of CS-specific cytotoxic T cell clones, which were shown to protect naive mice against challenge with sporozoites of *P. berghei* or *P. yoelii*, respectively (30, 32). More recently, a cytotoxic T cell epitope of the CS protein of *P. falciparum* was mapped using murine $CD8^+$ T cells, and the same epitope was shown to be recognized by human CTLs (23, 33).

Taken together, these findings suggest that the structural similarities of the CS proteins of distinct plasmodial species are matched by functional similarities. The findings also indicate that the CS proteins of different plasmodial species elicit similar antibody and cellular responses from their respective murine and human hosts. It is likely, therefore, that the two rodent malaria systems, *P. berghei* and *P. yoelii*, will continue to serve as useful experimental models for the investigation of the mechanisms of sporozoite-induced immunity, and of the role of the CS protein in protection.

Nevertheless, the marked differences in infectivity of sporozoites of distinct plasmodial species, and the inherent characteristics of the host's immune response, need to be taken into consideration when using the rodent models. Mice of different inbred strains vary in their susceptibility to sporozoite-induced *P. berghei* infection, and the susceptibility of mice to *P. yoelii* sporozoites is much greater than to *P. berghei* sporozoites. Inbred mice consistently develop an infection when injected with as few as 10 sporozoites of *P. yoelii*, while a 50 times greater number of *P. berghei* sporozoites is usually required to consistently produce patency. *P. berghei*, but not *P. yoelii*, sporozoites induce an early nonspecific inflammatory response in the liver (34, 35). While 20% of the iv injected *P. yoelii* sporozoites develop into liver stages in Balb/c mice, the corresponding

percentage of *P. berghei* sporozoites that develop into EEF is 0.01%. For these reasons, smaller challenging doses of *P. yoelii* sporozoites may be used in protection assays, probably mimicking more faithfully the situation in humans.

CS proteins of human and primate malarias: Polymorphisms in repeat and nonrepeat sequences Of the four species of malaria that infect humans, the CS genes of *P. falciparum* and *P. vivax* have been cloned and sequenced from numerous isolates, but only limited information is available with regard to the other two human plasmodial species, *P. malariae* and *P. ovale*. The sequence of the CS protein of *P. malariae* has, at this time, been established only for a single isolate (36). Except for three amino acid substitutions, located in different domains, the sequence of the CS protein of *P. malariae* is identical to that of *P. brasilianum*, a malaria parasite of several species of New World monkeys (37). This supported earlier immunological data on the sharing of the immunodominant epitope of the sporozoite surface antigen by these two closely related plasmodial species (38). The gene coding for the CS protein of *P. ovale*, a parasite which causes human malaria in limited areas of Africa, has not yet been cloned.

(i) Polymorphism within the repeat region. In contrast to the repeats of *P. falciparum*, recent isolates of *P. vivax* have been shown to contain a variant repeat region (39). Although the amino acid sequences outside the repeat domain were identical, the nonapeptide repeat (ANGAGNQPG) of several isolates from Thailand, designated VK247, shared only three amino acid residues with the earlier described repeat (GDRADGQPA) of the CS protein of *P. vivax* isolates from Brazil, El Salvador, North Korea, and Thailand. Monoclonal or polyclonal antibodies against the two types of vivax repeats were not cross-reactive. The VK247 variant has also been detected in isolates obtained from Papua New Guinea and Brazil (40), and antibodies against the (ANGAGNQPG) repeat have been detected in India, Colombia, and Cambodia, reflecting the wide geographic distribution of these parasites (41–43).

The occurrence of polymorphism in the repeats of the *P. vivax* CS protein was, in fact, not so surprising in view of the earlier reported antigenic diversity of the CS proteins of the *P. cynomolgi* complex (24, 25), a group of monkey malarias considered to be phylogenetically closely related to *P. vivax*. Examination of the antigenicity of the CS protein of 11 isolates of *P. cynomolgi* led to the conclusion that these isolates belonged to seven distinct groups, which displayed different immunodominant repeat B cell epitopes (25). The analysis of the nucleotide sequences of the CS genes of several *P. cynomolgi* isolates corroborated the immunological findings and revealed the presence of markedly different repeat-encoding

regions in five of the six isolates examined (44). In contrast to the variation found in the repeats, a strong homology (93–98%) was observed among the sequences flanking the repeats of the various *P. cynomolgi* isolates. These flanking regions were also highly homologous (90%) to the corresponding regions of the CS genes of the human malaria parasite, *P. vivax*.

The CS gene of *P. simium*, a parasite which only occurs in some monkey species in a limited region of Brazil, and which closely resembles *P. vivax* morphologically, has recently been cloned and sequenced. The repeat region of the CS protein of this parasite contained seven repeats of the "classical" *P. vivax* type, and two of the Thai variant (45).

Very recently, another variant of the CS protein of a *P. vivax*-like parasite was identified in a human blood specimen from Papua New Guinea. DNA of the vivax-like parasite hybridized with amplified fragments of the repeat region of the CS gene of *P. simiovale*, a monkey malaria parasite. Preliminary data indicate that this *P. vivax*-like parasite also occurs in Brazil and Indonesia (S. H. Qari, personal communication).

A nonhuman primate malaria with a CS protein sequence similar to *P. falciparum* has been described recently. The CS gene sequence of *P. reichenowi*, a *P. falciparum*-like malaria parasite of chimpanzees, has 83% similarity with the *P. falciparum* CS protein (46). It is remarkable that the predominant major repeat of *P. falciparum*, $(NANP)_n$, is the most frequent repeat of *P. reichenowi*, which also contains several copies of the minor repeat of *P. falciparum*, NVDP. The presence of the NANP, NVDP sequences in all *P. falciparum* isolates, and in *P. reichenowi*, taken together with the observation that all the nucleotide substitutions for these amino acids are synonymous, suggest an evolutionary pressure for conservation of these repeats. Most other differences between the CS sequences of *P. falciparum* and *P. reichenowi* concentrate in the areas surrounding the conserved regions I and II (46).

(ii) Polymorphism in nonrepeat regions of CS protein. The cloning of numerous CS genes over the past several years has demonstrated that different isolates of *P. falciparum* and *P. vivax* display relatively frequent substitutions of amino acids in well-defined, limited regions of the C terminal sequences of the CS protein.

Current data, based on *P. falciparum* isolates from different areas in Thailand, Papua New Guinea, and Brazil, indicate that the heterogeneity of the CS protein in these areas is considerably less frequent and more restricted than had been originally projected on the basis of the analysis of a limited number of African isolates. Indeed, among 23 *P. falciparum* isolates from different areas in the Amazon region, there was not a single

substitution in the CS protein sequence corresponding to a known polymorphic T cell epitope, Th3R (47). As for the domain in which another polymorphic T cell epitope is located, Th2R, the sequence of 19 of these isolates was identical to the sequence of 7G8, a Brazilian isolate originally sequenced by Dame et al (26). The amino acid sequences of Th2R of the other four isolates were identical, and they each differed from the 7G8 strain at the same five amino acid positions (47).

The analysis of a series of isolates from Papua New Guinea and Thailand resulted in similar conclusions, namely that the polymorphism in the C-terminal domains of the CS protein was very limited (48). It was rather striking that in the Papua New Guinea study, the sequences of all 22 Papua New Guinea isolates were found to be identical in the Th2R and Th3R T cell epitopes of the CS protein.

In another study, the CS sequences of *P. falciparum* isolates from a selected area of high malaria transmission in Papua New Guinea were found to be more polymorphic than Brazilian isolates obtained from an area with low malaria transmission (49). These findings suggest that the degree of polymorphism of CS gene may be related to the level of malaria transmission in a given area. Interestingly, all of the identified nucleotide substitutions in the codons for the Th2R region are nonsynonymous, indicating that there is strong selective pressure for change, while maintaining the general framework of the region. The Th2R and Th3R sequences flank region II, the ligand for the hepatocyte receptor. Perhaps the pressure for change derives from antibodies to Th2R and Th3R which would sterically hinder the interaction between ligand and receptor. Antibodies reacting with the Th2R and Th3R peptides have been detected in the sera of individuals living in endemic areas of Africa (50).

In the case of the *P. vivax* CS protein, a limited degree of polymorphism outside the repeat region has recently been reported, based on PCR amplification and sequence analysis of the CS genes of a relatively large number of isolates from Papua New Guinea and Brazil (51). Nonsilent nucleotide substitutions, of independent origin, were found to be limited to three domains of this protein which were analogous, in terms of location within the CS, with T cell epitopes that had been identified in the *P. falciparum* CS protein. The information currently available on T helper epitopes of the CS protein of *P. vivax* is rather limited, and putative cytotoxic epitope(s) of this protein have not yet been identified.

T CELL RESPONSE TO CS PROTEINS

T helper cell epitopes in the CS protein (i) Th epitopes within the repeat region. The identification of an immunodominant, protective, B cell

epitope within the repeat region of all CS proteins formed the basis for the design of first generation malaria subunit vaccines (see review—5). However, the analysis of the cellular responses of the volunteers involved in the first clinical trials of synthetic or recombinant *P. falciparum* CS vaccines, and subsequent studies using PBL of naturally infected individuals, indicated that the $(NANP)_n$ B cell epitope did not contain an immunodominant T cell epitope (Figure 3). Immunization of rodents with linear $(NANP)_n$ peptides induced T helper cells and elicited the production of antibodies only in $H–2^b$ mice (52, 53). These mice were also the only responder strain that developed antibodies following immunization with synthetic peptides containing multiple repeats of *P. vivax* (Thai strain), ANGAGNQPG, or repeats of the murine malarias, *P. berghei*, (DPPPPNPN), and *P. yoelii* (QGPGAP) (54–57). In the case of the *P. vivax* repeats, only $I\text{-}A^k$ mice developed anti-sporozoite antibodies when immunized with a truncated *P. vivax* CS protein containing the entire repeat region, or with an 18mer vivax repeat peptide (D/A) (58).

While the T cell response to the immunodominant B cell repeat epitope is limited, other T cell epitope(s) have been identified within the repeat region that may be more broadly recognized by immune T cells. Human T cell clones isolated from a *P. falciparum* sporozoite-immunized volunteer identified a unique epitope, NANPNVDPNANP, located within the 5′ minor repeat region that is present in all falciparum isolates (21). This epitope was also recognized by a high percentage of naturally infected individuals living in an endemic *P. falciparum* malaria area (C. Mason, unpublished). When incorporated into MAPs (multiple antigen peptides), the 5′ repeats induced T helper cells in mice of at least three haplotypes, $H–2^b$, $H–2^k$, and $H–2^a$ (59) and are therefore more widely recognized than the 3′ (NANP) repeats.

In the *P. vivax* CS protein, T cells that specifically recognize repeat region epitopes have been detected in immunized and naturally infected individuals. $CD4^+$ T cell clones, derived from a *P. vivax* sporozoite immunized chimpanzee, proliferated and secreted IL–2 and gamma interferon in response to challenge with a peptide containing the *P. vivax* repeat sequence (DRAAGQPAG)2 (60). PBL of naturally infected individuals living in South America frequently recognized a related repeat sequence, (GDRAAGQAA)2, as well as other peptides in the 3′ repeat region which contains a series of alternate or degenerate repeats (29). Lymph node cells of Balb/c mice, immunized with a pool of peptides representing the repeat region of the *P. vivax* CS protein, also proliferated in response to in vitro challenge with D/D repeat peptides and peptides representing the 3′ repeats of *P. vivax*.

(ii) T helper epitopes within nonrepeat regions. While a limited number

of T helper epitopes are present within the repeat region, the majority of T cell epitopes in the CS proteins of rodent and human malarias have been identified in regions outside the repeats (Figure 3).

(a) Murine malaria. Early studies, using cells obtained from mice immunized with recombinant CS protein, identified multiple T cell epitopes in the N and C terminal sequences of *P. berghei* CS protein (61). An N-terminal sequence, NEKIERNNKLKQP, and a C-terminal sequence, KQIRDSITEEWS, which include portions of the conserved regions I and II of the CS protein (underlined), induced T helper cells and high titers of anti-sporozoite antibodies when conjugated to a repeat peptide of *P. berghei*. The injection of peptides containing both the Th epitope and the repeats elicited a secondary antibody response in sporozoite-primed animals. The lymph node responses of peptide-primed mice demonstrated that the KQIRDSITEEWS epitope was recognized by at least four murine haplotypes, H–$2^{a,d,k,u}$. Incorporation of this Th epitope in a MAP vaccine, along with the *P. berghei* repeats, induced anti-sporozoite antibody titers in excess of 10^5 and protected the vaccine recipients against *P. berghei* sporozoite challenge (62, 63).

In the *P. yoelii* CS protein, a 21mer N-terminal peptide, aa 59–79, selected on the basis of an amino acid sequence motif commonly found in murine and human T cell epitopes, induced proliferation in lymph node cells of peptide primed H–2^d and H–2^b, but not H–2^k, mice (57). $CD4^+$ T cell clones of both Th1 and Th2 subsets were derived from peptide immunized mice of both responder haplotypes. The *P. yoelii* $CD4^+$ T cell clones were species-specific and did not recognize the homologous peptide sequence of the *P. berghei* CS protein, which differs at six amino acid positions. Peptide aa 59–79 coupled to the *P. yoelii* repeats induced T helper cells in H–2^d mice, a strain of mice which is a nonresponder when immunized with repeat peptide alone.

(ii) Human malaria. Studies carried out using cells obtained from naturally infected individuals have also identified multiple T cell epitopes in the nonrepeat regions of *P. falciparum* and *P. vivax* CS proteins. PBL of Africans, living in an area of endemic *P. falciparum*, frequently recognized an epitope contained in the amino acid sequence,

PSDKHIEQYLKKIKNSIS,

which is located in a C-terminal polymorphic region of the *P. falciparum* CS protein (28). Previous studies in mice had shown that this amino acid sequence, which was predicted to be a T cell epitope based on a high amphipathic alpha helix index, was a T helper epitope (Th2R) recognized by mice expressing I-A^k class II MHC molecules (64). The PBL of a large number of West Africans also proliferated in response to challenge with

another C-terminal polymorphic epitope Th3R, contained in the aa sequence IKPGSANKPKDELDYENDIE (28). Recognition of the Th3R epitope appeared to correlate with resistance of East Africans (65), but not West Africans (66), to reinfection with *P. falciparum*. More recent studies have shown a different pattern of response, when the same set of overlapping peptides representing the entire *P. falciparum* CS protein was used to test PBL obtained from individuals living in areas of lower malaria endemicity in Papua New Guinea (67).

Algorithms based on amphipathicity and amino acid motifs have been used to predict an additional T cell epitope in the *P. falciparum* CS protein, designated CS.T3 (68). This epitope, contained in the peptide sequence DIEKKIAKMEKASSVFNVVNS, mapped to a conserved region of the *P. falciparum* CS protein. T cells clones specific for the synthetic peptide representing this sequence, in which alanines (underlined) were substituted for the cysteines found in CS protein, also recognized the antigen in *P. falciparum* sporozoite extracts. The CS.T3 peptide sequence was found to be a "universal" epitope in that it could bind to numerous DR molecules and stimulate proliferation of T cells restricted by a broad range of class II molecules (69).

A second epitope that is also presented by multiple class II DR molecules has recently been defined in amino acid sequence 326–345 of the NF54 isolate of *P. falciparum* (22; A. Moreno, submitted). This epitope, EYLNKIQNSLSTEWSPCSVT, which contains part of region II (underlined), was identified by screening clones obtained from sporozoite-immunized volunteers. The same epitope was found to stimulate class II–restricted cytotoxic and noncytotoxic human T cell clones, as well as murine T helper cells (E. Nardin, unpublished information) and is therefore referred to as Pf Th/Tc. A similar sequence in the 7G8 strain was found to stimulate $CD45Ra^+$ "naive" T cells obtained from nonimmune individuals (70).

The Pf Th/Tc epitope is unique in that it overlaps not only the conserved Region II of the CS molecule but also part of the Th2R polymorphic region of the CS protein. The $CD4^+$ T cell clones specific for the Pf Th/Tc epitope do not recognize the Th2R peptide (A. Moreno, unpublished information). Epitope mapping has shown that amino acids from region II, in addition to a variable number of amino acids in the polymorphic domain, are required for recognition of the Pf Th/Tc epitope by the human $CD4^+$ T cell clones.

On the basis of limited studies it has been speculated that the polymorphism in the Th2R region plays a role in the evasion of the host's immune response by the parasite. These studies demonstrated that cells obtained from mice immunized with synthetic peptides and T cells from non-immune humans had limited cross-reactions with polymorphic TH2R

sequences (71–74). However, subsequent studies in Africa indicated that PBL of naturally infected individuals living in areas of high endemicity frequently recognized peptides containing variants of the polymorphic sequences of the Th2R peptide (66, 75). A total of 13/30 of the Th2R responders reacted to all three strain variants of the Th2R epitope that were tested (66). Human $CD4^+$ T cell clones, specific for the Pf Th/Tc epitope that overlaps this polymorphic region, have recently been shown to recognize a large number of variant peptides that correspond to polymorphisms detected in *P. falciparum* isolates from Africa, Asia, and South America (A. Moreno, P. Clavijo, R. Edelman, J. Davis, M. Sztein, F. Sinigaglia, E. Nardin, submitted for publication).

In the case of the *P. vivax* CS protein, multiple T cell epitopes have also been identified in the sequences that flank region II. T cell lines obtained from a *P. vivax* sporozoite immunized chimpanzee have identified an epitope, EYLDKVRATVGTEWTPCSVT (Figure 3, chT), which preceeds and includes part of region II (60). The chT epitope is analogous, in terms of location, to the Pf Th/Tc epitope in the *P. falciparum* CS protein identified by the T cell clones derived from the *P. falciparum* sporozoite-immunized human volunteers.

PBL obtained from naturally infected individuals, living in an area of endemic *P. vivax* malaria in South America, predominantly recognized either the aa sequences that follow the repeats, or sequences that follow the cysteine pairs of region II (29). The highest number of responders (15/26) was obtained with the peptide sequence,

VRRRVNAA/TNKKPEDLTLNDL

that follows region II in the *P. vivax* CS protein.

Lymph node cells of Balb/c mice immunized with peptide pools, or with a recombinant *P. vivax* CS protein, have identified additional epitopes in the region II domain. A synthetic peptide TVGTEWTPASVTA, in which alanine was substituted for the cysteines of region II, stimulated maximal proliferative response in lymph node cells of $H\text{–}2^b$ mice immunized with a recombinant *P. vivax* CS protein (76). Overlapping peptides spanning a similar region, RATVGTEWTPCSVTCGV/AGVRVRRRVNAA/TNK also stimulated proliferation of lymph node cells obtained from mice immunized with peptides representing the C-terminus of *P. vivax* CS protein (29, 77). Peptides from this region of the *P. vivax* CS protein contain a cell adhesion motif in the VTCG sequence (underlined above), that has been shown to bind human hematopoietic cell lines (78).

The recent analysis of over 115 CS clone sequences, representing 15 isolates from Papua New Guinea and 24 isolates from Brazil, has identified polymorphic amino acid positions in the *P. vivax* CS protein (51). The

sequences following region II, which were frequently recognized by PBL of naturally infected individuals, contained two polymorphic positions at aa 341 (V/A) and 350 (A/T). The epitope located immediately after the repeats, NNEGANAP/L/TN/SEKSVK/IEYLDKV, which was recognized by 33% of the PBL tested, also contained polymorphism at three aa positions. However, no aa changes were observed in an N-terminal epitope that was also frequently recognized by PBL of individuals living in the vivax endemic areas. In addition, no changes were detected within the T cell epitope identified by T cells of a *P. vivax* sporozoite immunized chimpanzee.

The function of these T cell epitopes in the immune response to the *P. vivax* CS protein remains to be defined. In the *P. falciparum* CS protein, all the T cell epitopes identified by the proliferative response of immunized or naturally infected individuals have been shown to function as T helper cells in the production of anti-sporozoite antibodies. The combination of any of the C terminal T cell epitopes defined thus far, Th2R, Th3R, CS.T3 or Pf Th/Tc, with CS repeats in MAPs, induced anti-sporozoite antibodies in murine strains that fail to respond to immunization with the *P. falciparum* repeats alone (M. Calvo Calle, G. de Oliveira, P. Clavijo, M. Maracic, J. Tam, E. Nardin, R. Nussenzweig. A. Cochrane, submitted). In addition, one of the T helper epitopes, Pf Th/Tc contained in aa 326–345, is also recognized by cytotoxic $CD4^+$ T cells, as discussed below.

Cytotoxic T cell epitopes in the CS protein The efficacy of the protective humoral responses targeted to the extracellular, infective sporozoite depends on the concentration and fine specificity of the anti-sporozoite antibody response (62, 63, 79). However, in addition to humoral immunity, cell-mediated immune responses of the sporozoite-immunized host also protect against sporozoite challenge. CS specific $CD8^+$, as well as $CD4^+$, T cells can inhibit the intracellular EEF, as it develops within the host hepatocytes.

(i) $CD8^+$ CTL epitopes. Elimination of $CD8^+$, but not $CD4^+$, T cells in sporozoite-immunized mice results in the loss of protection against sporozoite challenge in *P. berghei*–immunized A/J, or *P. yoelii*–immunized Balb/c mice (80, 81). $CD8^+$ T cells, derived from sporozoite-immunized Balb/c mice, can inhibit EEF development in hepatocytes and lyse peptide-pulsed target cells in a class I–restricted manner (30, 31, 82). The H–2 K^d restricted murine CTL clones recognized similar sequences in the CS protein of each malaria species, aa sequence NDDSYIPSAEKI in *P. berghei* (30) and NEDSYVPSAEQI (32), or SYVPSAEQILEFVKQI (31) in the *P. yoelii* CS protein (Figure 3). Analysis of the fine specificity of the rodent $CD8^+$ CTL clones indicated that two residues in each sequence,

tyrosine and isoleucine, are critical aa for interaction with the class I H–$2K^d$ molecule (83).

The murine CS-specific $CD8^+$ T cells clones, when passively transferred to naive mice, protected the recipients against challenge with viable sporozoites of the homologous species (30, 32). Although both CTL epitopes are located in similar regions of the C-terminus of the respective CS proteins, and differ by only three amino acids, the response of most *P. yoelii* clones was species-specific. However, cross-reactivity in vitro and partial protection in vivo was obtained with some *P. berghei* $CD8^+$ CTL (32). Protection was the result of inhibition of parasite EEF development. The levels of parasite ribosomal RNA in the livers of recipients of protective clones were $< 5\%$ of controls when measured using oligonucleotide probes specific for *P. yoelii* rRNA (84).

In the *P. falciparum* CS protein, a $CD8^+$ CTL epitope was identified using murine T cells obtained from B10.BR ($H\text{–}2^k$) mice immunized with a vaccinia vector expressing the entire falciparum CS protein (33). This C-terminal epitope, aa 368–390 of the 768 strain,

KPKDELDYENDIEKKICKMEKCS,

was also recognized by $CD8^+$ T cells obtained from mice immunized with *P. falciparum* sporozoites. Notably, the same epitope sensitized target cells for lysis by $CD8^+$ T cells derived from three out of four sporozoite-immunized volunteers (23). This epitope was recognized by both protected and nonprotected volunteers. Although this region of the CS protein is polymorphic, both murine and human CTL specific for peptide 368–390 of the 7G8 strain lysed target cells sensitized with the NF54 strain peptide, which differs from 7G8 in a single aa substitution (E/A at position 376).

(ii) $CD4^+$ CTL epitopes. While depletion of $CD8^+$ T cells resulted in loss of sporozoite-induced immunity in Balb/c mice, depletion of $CD8^+$ T cells in other strains of mice did not affect the level of protective immunity against *P. yoelii* sporozoites (85). Furthermore, the depletion of $CD8^+$ T cells from A/J (81) but not Balb/c mice (86) led to loss of immune resistance in mice vaccinated with irradiated *P. berghei* sporozoites.

Class II–restricted $CD4^+$ CTL have been detected in both murine and human hosts during bacterial and viral infections (87–89). Sporozoite antigens, including the CS protein, also sensitize $CD4^+$ T cells that can inhibit intracellular EEF development both in vitro and in vivo. A synthetic peptide containing a T helper epitope, located in the N-terminal of *P. yoelii* CS protein, elicited $CD4^+$ T cells that destroyed *P. yoelii*-infected hepatocytes in vitro (90). The passive transfer of $CD4^+$ clones specific for this epitope protected naive recipients against *P. yoelii* sporozoite challenge. A cytotoxic $CD4^+$ CTL clone from *P. berghei* sporozoite-

immunized mice, which recognizes a non-CS antigen shared by sporozoites and blood stages, lysed antigen-pulsed target cells in vitro (90). Passive transfer of this clone protected naive animals from challenge with *P. berghei* sporozoites, but not with blood stages of the parasite.

Human $CD4^+$ cytotoxic T cell clones have recently been derived from three sporozoite-immunized volunteers who were protected against *P. falciparum* sporozoite challenge (22; A. Moreno, submitted for publication). These class II–restricted $CD4^+$ CTL lyse EBV-transformed B cells pulsed with a peptide representing a C-terminal sequence of the *P. falciparum* (NF54) CS protein. The epitope recognized by the cytotoxic $CD4^+$ T cells is contained in aa 326–345 in the CS protein of the NF54 strain, EYLNKIQNSLSTEWSPCSVT, which contains a Th epitope described above (Pf Th/Tc). The class II–restricted $CD4^+$ CTL epitope is distinct from the epitope recognized by the class I–restricted $CD8^+$ CTL, KPKDELDYANDIEKKICKMEKCS (33). Peptides representing the $CD4^+$ CTL epitope, which overlapped part of the polymorphic as well as the conserved region II (underlined) of the *P. falciparum* CS protein, stimulated cytotoxic as well as noncytotoxic human $CD4^+$ T cell clones (A. Moreno, in preparation). Multiple DR haplotypes were found to effectively present the Pf Th/Tc peptide for proliferative and cytotoxic responses in vitro.

Non-CS Sporozoite Antigens Involved in Protective Immunity

SPOROZOITE SURFACE PROTEIN 2 (SSP2) Immunization with sporozoites predominantly induces an antibody response to the repeat region of the CS protein. However, additional antibody specificities have been identified using monoclonal antibodies derived from *P. yoelii* sporozoite-immunized mice. One monoclonal antibody, NYS4, gave a patchy pattern of IFA reactivity with internal and surface antigens when tested against air-dried *P. yoelii* sporozoites (92). The NYS4 antibody, which did not neutralize sporozoite infectivity or give a CSP reaction with viable sporozoites, reacted with a 140-kDa protein in Western blots of sporozoite extracts. Screening of a *P. yoelii* gene expression library with this monoclonal antibody led to the identification of the "sporozoite surface protein 2" (SSP2) gene (93). The deduced SSP2 protein has some features in common with CS protein, including the presence of repeats, and an N-terminal sequence, EEWSECSTTCDEGRK, with homology to region II of the *P. yoelii* CS protein (identical amino acids underlined).

$CD8^+$ CTL specific for both *P. yoelii* SSP2 and CS are induced by sporozoite immunization in Balb/c mice (94). The epitope recognized by the SSP2 $CD8^+$ CTL has not yet been defined. The SSP2 specific CTL lysed tumor target cells transfected with a SSP2 gene fragment lacking any

sequence similar to the $CD8^+$ CTL epitope defined in the CS protein (31, 32). A limited number of mice (3/6) immunized with histocompatible P815 tumor cells transfected with the SSP2 gene were protected against *P. yoelii* sporozoite challenge. When tumor cells expressing either SSP2 or CS were both used for immunization, all of the mice were protected (94). Immune resistance in the mice immunized with SSP2−, or the CS− transfected tumor cells, was abrogated by depletion of $CD8^+$ T cells prior to challenge.

The demonstration of the role of SSP2 in protective immunity has led to the search for an homologous antigen in *P. falciparum* sporozoites. The *P. yoelii* SSP2 protein has amino acid sequence homology with a *P. falciparum* blood stage antigen, the "thrombospondin related anonymous protein" (TRAP) (13). In the sequence with region II homology, 32/62 amino acids are identical including complete concordance of 7 cysteines. While *P. falciparum* TRAP lacks the repeats found in *P. yoelii* SSP2, the predicted aa sequences of each protein share 43% similarity in the amino terminal 281 aa and 56% similarity in the carboxy terminal 71 amino acids. TRAP is also expressed in sporozoites and liver stages of the parasite and recent studies have characterized the TRAP antigen as the SSP2 homologue of *P. falciparum* (95).

Immunoelectron microscopy localized the expression of TRAP primarily in the micronemes, and to a limited extent on the surface, of *P. falciparum* sporozoites (95a). Anti-TRAP antibodies inhibited *P. falciparum* sporozoite invasion of hepatocytes in vitro. PBL of 3 out of 5 *P. falciparum* sporozoite-immunized volunteers with no history of blood stage infections proliferated in response to recombinant TRAP/SSP2, and 2 out of 4 volunteers had antibodies that reacted with the protein.

The ability of mice immunized with *P. falciparum* sporozoites to resist challenge with *P. berghei* sporozoites has led to the suggestion that protective antigens may also exist that are shared between these two species of Plasmodia (96). Polyclonal and monoclonal antibody obtained from mice immunized with *P. falciparum* sporozoites reacts with a 42 kDa antigen, termed CSP–2, in extracts of *P. berghei* and *P. falciparum* sporozoites. The monoclonal anti-CSP–2 antibody does not react with repeat sequences of the CS protein of either species of malaria.

PROTECTIVE ANTIGEN SHARED BY BLOOD STAGES AND SPOROZOITES In addition to the CS and SSP2 antigens that are expressed in sporozoite, EEF, and erythrocytic stages, a protective antigen shared by sporozoites and blood stages of *P. berghei* and other Plasmodial species has also been demonstrated. A $CD4^+$ CTL clone, obtained from mice immunized with *P. berghei* sporozoites, lysed $I\text{-}E^{d+}$ target cells pulsed with extracts of sporozoites or blood stage parasites (91). This T cell clone can also be

stimulated by extracts of blood stages of *P. berghei*, *P. yoelii*, *P. chabaudi* and also *P. falciparum*. Passive transfer of this clone protects naive Balb/c mice against challenge with *P. berghei* sporozoites, but not blood stages. The antigen recognized by the class II–restricted $CD4^+$ CTL has not yet been characterized, but it is distinct from the CS protein, since the clone failed to proliferate or to secrete gamma interferon in the presence of CS protein or overlapping synthetic peptides corresponding to this protein.

Liver Stage-Specific Antigens

LSA–1 AND LSA–2 Parasite antigens specific for the exoerythrocytic forms have been identified as potential targets of immunity. Liver stage antigens (LSA) of the malaria parasite were first defined using serum obtained from an individual who had lived for 26 years in an area of hyperendemic *P. falciparum* malaria while under constant chemoprophylaxis (97). The serum, which had high titers with sporozoites and liver EEF, but neglible reactivity with infected red blood cells, was used to screen a genomic expression library of *P. falciparum* DNA from a Thailand isolate. An antigen expressed only by EEF was described, LSA–1, which contained a 17-residue repeat, EQQSDLEQERLAKEKLQ. The LSA–1 gene of NF54 strain of *P. falciparum* encodes a 230 kDa acidic protein containing 86 copies of the 17 aa repeat sequence (98). Antibodies generated against the repeat peptide of LSA–1 reacted with flocculent material within the parasitophorous vacuole of *P. falciparum* EEF (96). Although antibodies against LSA repeats were detected in the sera of naturally infected individuals, PBL of individuals living in endemic areas did not proliferate in response to the repeat peptide, suggesting a lack of T cell epitopes. Conjugation of the LSA repeats to the Th2R epitope of the CS protein induced anti-repeat antibodies in $H\text{–}2^d$ as well as $H\text{–}2^k$ mice, and these antibodies reacted with the native molecule in the EEF (99).

Antibody to the repeat, but not the nonrepeat, sequences of *P. falciparum* LSA–1 cross-reacted with a 230 kDa antigen of *P. berghei* EEF, termed LSA–2 (96). This *P. berghei* liver stage antigen was localized by confocal microscopy on extensions of the parasitophorous vacuole membrane (PVM) that form a membranous network in the cytoplasm of *P. berghei*-infected hepatocytes (100). A monoclonal antibody specific for *P. berghei* EEF recognizes an antigen, designated PbLl, that also appears to localize to the parasitophorous membrane (101).

Mice immunized with the LSA repeat peptide were protected against challenge by the bite of 10 *P. berghei*-infected mosquitoes (96). In preliminary experiments, spleen cells from protected mice were able to kill up to 80% of *P. berghei*-infected hepatocytes in vitro.

HEAT SHOCK PROTEINS Erythrocytes and hepatocytes infected with *P. falciparum* express heat shock proteins (102, 103). Monoclonal antibody raised against a C-terminal region of the 72kDa protein of *P. falciparum* (Pfhsp70) was found to function in antibody-dependent cell cytotoxicity (ADCC) (104). In the presence of this monoclonal antibody, non-parenchymal liver cells destroyed *P. yoelii* EEF in mouse hepatocyte cultures at effector: target cell ratios of 10: 1. The effector cells within the nonparenchymal cell population were believed to be FcR^+ Kupffer cells.

In another series of experiments, monoclonal antibodies against the plasmodial hsp 70 were generated by the repeated immunization of mice with *P. berghei* infected RBCs (M. Tsuji, in preparation). The binding sites of these monoclonal antibodies were mapped to the C-terminal and central regions of the molecule, using truncated recombinants of the *P. falciparum* hsp70. These anti–heat shock antibodies reacted with air-dried sporozoites of *P. berghei*, *P. yoelii*, *P. falciparum*, and *P. vivax*, but not with live or glutaraldehyde-fixed sporozoites. They also react with EEF and blood stages of Plasmodia. A $CD4^+$ T cell clone, obtained by immunization with *P. yoelii* sporozoites, proliferated upon stimulation with Pf hsp 70 or infected red blood cells. Passive transfer of this T cell clone, which is of the TH–2 type, failed to confer protection against sporozoite challenge.

MECHANISMS OF IMMUNITY TO EXOERYTHROCYTIC FORMS OF MALARIA

Direct Cytotoxicity

The direct interaction of $CD8^+$ CTL with target cells results in cell lysis and/or apoptosis (Figure 2). All of the murine CS-specific $CD8^+$ CTL contain perforin granules and serine esterases. However, in the rodent *P. yoelii* model, not all of the CS specific $CD8^+$CTL clones were protective in vivo, although they had the same fine specificity and lytic activity in vitro. The ability of CTL to passively protect naive recipients correlated with the expression of high levels of an adhesion molecule, CD44, as well as VLA–4, on the surface of the protective $CD8^+$ CTL clones (105). Based on autoradiograms of liver sections made 4–8 hr after the injection of radiolabelled CTL clones into infected mice, there was a correlation between the $CD44^+$ phenotype of the clones and their ability to make contact with the EEF-infected hepatocytes. CTL clones expressing low levels of adhesion molecules were found in the liver after passive transfer, but failed to localize to the infected hepatocytes.

In contrast to the ubiquitous expression of class I molecules on target cells, the lack of class II molecules on liver parenchymal cells might limit

the ability of $CD4^+$ CTL to interact directly with EEF-infected hepatocytes. Nevertheless, while class II molecules are not constitutively expressed on hepatocytes, HLA-DR molecules can be induced in the course of viral infections and during transplantation reactions (106–108). Perhaps this also occurs in malaria-infected hepatocytes, because the ability of murine CS-specific $CD4^+$ T cell clones to destroy EEF in vitro, and to passively protect naive recipients in vivo, is class II restricted (90).

Lymphocytes can also induce the expression of class II HLA molecules on hepatocytes (108). It is conceivable that gamma interferon, secreted by T cells activated by recognition of CS or other parasite antigens presented by class II–positive Kupffer cells, could induce the expression of class II on the neighboring EEF-infected hepatocytes.

Cytokine Mediated Immune Resistance

In addition to direct lysis of target cells, T cells may block EEF development indirectly through the induction or secretion of inhibitory lymphokines or cytokines (Figure 2). Gamma interferon was the first T cell–derived lymphokine shown to have an inhibitory effect on EEF of malaria (109, 110). While gamma IFN has a pleotrophic effect on immune functions, its ability to inhibit EEF development in hepatoma cells in vitro, in the absence of other cell types, suggests that the lymphokine acts directly on the infected hepatocyte. The administration of anti-gamma interferon to *P. berghei* sporozoite-immunized mice abrogated immune resistance to sporozoite challenge (81).

Other lymphokines, such as IL–6, TNF, and IL–1, also inhibit the development of EEF in vitro and in vivo (111–114). Because gamma interferon, TNF, and IL–1 can induce IL–6 production by liver parenchymal and/or non-parenchymal cells, these cytokines may act in concert to prevent parasite development in the liver.

The ability of lymphokines, IL–6, and gamma interferon to inhibit EEF development has been shown to be L-arginine dependent (115–116). Nitric oxide (NO) is generated from L-arginine by oxidation of a terminal nitrogen in the guanidino group, a reaction catalysed by NO synthase. Reactive nitrogen intermediates, in particular NO, play a role in the destruction of intracellular protozoan parasites by gamma interferon activated macrophages (117–118).

Hepatocytes generate high levels of NO following stimulation with lymphokines or supernatants obtained from activated Kupffer cells (119–120). Human hepatocytes produced significant levels of NO endproducts, nitrite and nitrate, when stimulated with a combination of cytokines TNF/IL–1/IFN, while single cytokines were ineffective (119). Lymphokine stimulation of macrophages or hepatocytes induce transcription and trans-

lation of the inducible NO synthase (iNOS) gene, which has been recently cloned in human hepatocytes (A. Nussler, submitted) and murine macrophages (121).

A competitive inhibitor of NO synthesis, a guanidino methylated derivative of L-arginine N^Gmonomethyl-L-arginine (N^GMMLA), binds more efficiently to NO synthase than does L-arginine and thus inhibits production of NO. The addition of N^GMMLA to primary cultures of mouse hepatocytes reversed the inhibitory effects of gamma interferon on *P. berghei* EEF development (115). The ability of TNF and IL–6 to inhibit *P. yoelii* EEF development in Balb/c primary cultures or co-cultures was also abrogated by L-arginine analogues, N^GMMLA and Nω-nitro-L-arginine (NωNLA) (116).

IL–6 and the related cytokines IL–1 and TNF are also potent inducers of hepatocyte synthesis of acute phase proteins. A major acute phase reactant, the C-reactive protein (CRP), binds to sporozoites and inhibits the early stages of EEF development (122). Rats with high levels of CRP in their circulation, induced by turpentine injection, were resistant to infection with *P. yoelii* sporozoites. However, transgenic mice with constitutive high levels of CRP in their circulation were fully susceptible to *P. yoelii* sporozoite-induced infection when compared to normal controls (L. Renia, submitted for publication).

VACCINE DEVELOPMENT

Design of Subunit Vaccines Containing T and B Cell Epitopes

Taken together, the data on humoral and cell-mediated immunity indicate that a multiplicity of protective mechanisms are induced by sporozoite immunization. Therefore, malarial vaccine design will require multiple T and B cell epitopes incorporated into constructs and adjuvants which present, in an effective manner, a combination of antigens to the human immune system.

CHOICE OF TH EPITOPES FOR SUBUNIT VACCINES The low antibody responses in the first series of clinical trials of CS based *P. falciparum* vaccines were due, in part, to vaccine design and inadequate adjuvant formulations. Carrier proteins, tetanus toxoid (TT) and tet32 (32 amino acids of a tetracycline resistance gene read out of frame), were used to provide T helper epitopes in each of the first generation repeat based vaccines (123, 124). Subsequent studies have shown that Balb/c mice immunized with recombinant fusion protein, R32tet32, developed suppressor cells specific for the tet32 carrier, which inhibited the anti-NANP repeat (R32) antibody response in R32tet32 immunized mice (125).

Recent vaccine designs, based on the *P. falciparum* (NANP) repeats, have addressed the problems of genetic restriction of the response to CS repeats and the use of bacterial proteins as carriers for peptide vaccines. Bacterial proteins such as tetanus toxoid have recently been shown to contain T cell epitopes which are "universal," i.e. are recognized by T cells of a large number of individuals, using multiple class II molecules as restriction elements (126). The incorporation of universal Th cell epitopes in vaccines would ensure the induction of an immune response in the majority of immunized individuals. The (NANP)40-PPD conjugate induced high levels of anti-sporozoite antibodies in BCG primed mice of different haplotypes without the use of adjuvants (127).

The efficacy in humans of peptide vaccines in which the carrier protein is BCG, tetanus or diphtheria toxoid may be hindered by epitopic suppression, that is the inhibition of the antipeptide antibody response by the carrier-specific immune response induced by prior vaccination (128–131). In the case of NANP-TT vaccine, all vaccinees had been previously vaccinated with TT, and anti-TT immune responses may have inhibited the response to NANP (132, 133). In order to circumvent this problem, a "universal" tetanus toxoid T cell epitope, TT_{73-99}, which is devoid of B cell epitopes, has been used as a carrier for (NANP)4 (134). In TT-primed mice, the administration of (NANP)4-TT_{73-99} conjugate induced high levels of anti-repeat antibody. In contrast, little or no antirepeat antibody was detected in the sera of TT-primed mice following administration of (NANP)3-TT.

A limitation of vaccines containing "universal" peptides from foreign proteins is the lack of parasite-derived T cell epitopes that are required to induce cell-mediated immunity and anamnestic immune responses following natural exposure to the bites of infected mosquitoes. A universal peptide has been defined in the repeat region of the *P. malariae* CS protein, which is recognized by most murine strains (135). MAPs containing both the *P. malariae* and the *P. falciparum* repeats induced an anti-repeat antibody response in all strains of mice tested. However, this *P. malariae* Th epitope would also fail to induce the species-specific T cell response required for an effective immune response to *P. falciparum* sporozoite challenge.

The *P. falciparum* CS protein contains at least two epitopes, CS.T3 and Pf Th/Tc, that can be presented by multiple genetic restriction elements. Synthetic vaccines containing CS.T3, conjugated to the repeat B cell epitope $(NANP)_n$, induced anti-repeat antibodies in the majority of murine strains tested (69; M. Calvo Calle, submitted). The CS.T3 epitope also provided efficient help for an antibody response to blood stage antigen of *P. falciparum* (136).

Similar to the CS.T3 epitope, the Pf Th/Tc epitope, contained in aa 326–345 (NF54), binds to multiple class II molecules (DR 1, 2, 4, 7 and 9) (A. Moreno, submitted). When combined with *P. falciparum* repeat peptide in a MAPs construct, this epitope can induce anti-sporozoite antibody responses in Balb/c, C3H, A/J and C57Bl mice. While the Pf Th/Tc epitope overlaps a region of the *P. falciparum* CS protein that is highly polymorphic, human $CD4^+$ T cell clones specific for this epitope recognized the majority of peptides representing the polymorphic sequences of *P. falciparum* isolates from Africa, Asia, and South America. The cross-reactivity of these T cells clones suggests that polymorphism in this region of the CS would not preclude inclusion of the Pf Th/Tc epitope in a vaccine.

Although the antibody response to linear peptides containing the T epitopes Th2R and Th3R, when combined with the *P. falciparum* repeats, is restricted to one or a few haplotypes (137, 138), the ability of these T epitopes to elicit helper cells in other strains can be enhanced by vaccine design. Incorporation of the Th2R epitope with (NANP) repeats within MAPs (M. Calvo Calle, submitted for publication), or in liposomes (139), induced antibody responses in strains of mice that failed to respond to linear peptide conjugates. Furthermore, (NANP)40 contained in a tetra-branched MAP construct, but not the (NANP)40 linear peptide, could induce anti-repeat antibody response in nonresponder strains of mice (140). However, the incorporation of other repeats into similar MAPs constructs did not overcome the genetic restriction of the response to *P. yoelii* or *P. vivax* repeat peptides.

It remains to be determined which combination of T and B cell epitopes will induce optimal levels of humoral and cell mediated immunity in humans. MAPS containing the *P. falciparum* 5′ repeat T helper epitope, (NDPNANPNVDPNANPNV), combined with the (NANP)3 B cell epitope, induced IFA titers of over 1 million in responder $H\text{–}2^b$ mice, and over 10^5 in intermediate responder strains, $H\text{–}2^a$ and $H\text{–}2^k$ mice (59). MAPs containing other T cell epitopes, Th2R, Th3R, or CS.T3, in combination with NANP, induced peak IFA titers of 10^4 in mice that were responders to the Th epitope (M. Calvo Calle, submitted). However, an analysis of the anti-sporozoite and anti-peptide antibody responses induced by MAPs containing a variety of T cell epitopes in various orientations has not identified a consistent MAPs geometry and composition that would predict the level or fine specificity of the immune response.

VACCINES CONTAINING $CD8^+$ CTL EPITOPES In contrast to the multiple epitopes that are available for class II–restricted T cell recognition, only a single class I–restricted $CD8^+$ T cell epitope in the *P. falciparum* CS proteins has been defined. The antigen processing pathways are distinct for

the exogenous antigens, which are usually presented to class II–restricted $CD4^+$ T cells, versus the endogenous antigens, presented to class I–restricted $CD8^+$ T cells. Therefore, vaccines containing $CD8^+$ CTL epitopes must be designed to target the antigen to the correct intracellular processing pathway for the formation of the class I/antigen complexes necessary for $CD8^+$ T cell stimulation.

One means of targeting CS for class I presentation is to use recombinant bacterial or viral vectors to express the CS protein intracellularly. The $CD8^+$ CTL epitope in *P. falciparum* CS protein was originally defined using cells obtained from mice immunized with a recombinant vaccinia virus expressing CS (33). The same epitope specificity was induced in human volunteers immunized with *P. falciparum* sporozoites (23).

Bacterial vectors have also been used to induce CS specific $CD8^+$ CTL in immunized mice. Oral immunization with an attenuated *Salmonella typhimurium* transformed with a plasmid expressing the CS gene of *P. berghei* induced $CD8^+$ T-cell–dependent immune protection in 55–70% of immunized Balb/c mice (141, 142). Anti-CS antibody was not detected following per os immunization with the *S. typhimurium* transformants. $CD8^+$ T cells derived from the spleens of immunized mice specifically lysed target cells pulsed with the *P. berghei* $CD8^+$ CTL epitope,

NDDSYIPSAEKI.

Mice immunized with aroA *S. typhimurium* mutants expressing the *P. yoelii* CS protein also developed CS-specific $CD8^+$ CTLs (143). However, the mice immunized with the Salmonella/*P. yoelii* recombinant were not protected against *P. yoelii* sporozoite challenge.

Recent studies have shown that exogenous as well as endogenous CS antigen can induce murine $CD8^+$CTL. A 9mer peptide, containing a $CD8^+$ T cell epitope of the *P. berghei* CS protein, when modified by the addition of a palmitoyl-cys-ser lipid tail, induced murine $CD8^+$ T cells specific for the parasite (83). Lipopeptides have also been used to induce $CD8^+$ CTL specific for viral antigens (144). The addition of the lipid tail was believed to target the peptide to the cytoplasm of antigen presenting cells where it was processed as an endogenous antigen.

Synthetic peptides without the lipid tail can also induce $CD8^+$ CTL specific for the *P. berghei* CS protein. These peptides contained T helper epitopes that overlapped with the $CD8^+$ CTL epitopes or were constructed to contain both $CD4^+$ T helper and $CD8^+$ CTL epitopes (83). In fact, the development of $CD8^+$ T cell–mediated immunity in *P. yoelii* immunized Balb/c mice is $CD4^+$ T cell dependent (145). The presence of a Th epitope in the Py1 21mer peptide of the *P. yoelii* CS protein may have played a

role in the ability to induce CS specific $CD8^+$ T cells in mice immunized with this peptide (90).

Exogenous antigens can also induce class II–restricted $CD4^+$ CTL specific for different viral antigens (146, 147). The Pyl peptide, representing the N-terminal sequence of *P. yoelii* CS protein, elicits $CD4^+$ T cells as well as $CD8^+$ T cells (90). The $CD4^+$ T cells, derived from those peptide-immunized mice, inhibited EEF development in vitro and were protective when passively transferred to naive recipients. However, the peptide-immunized mice themselves were not protected against challenge.

Although the transfer to naive mice of CS-specific $CD4^+$ or $CD8^+$ T cell clones protects them against malaria, it has been difficult to obtain consistently high levels of T cell–mediated protective immunity by vaccination with recombinant or synthetic peptide CS vaccines. The number of CTL induced by immunization may not be sufficient to protect against sporozoite challenge. Total protection against sporozoites may require CTL directed at two or more parasite antigens, as has been shown with the *P. yoelii* CS and SSP2 antigens. It is also possible that the vaccines may not elicit CTL with the high CD44/VLA–4 phenotype which may be required for effective targeting to the EEF in the liver. The synergistic effects of antibody, to reduce the number of sporozoites that can infect hepatocytes, combined with CTL to destroy a low number of EEF, may also be necessary for total protection of the sporozoite-immunized host in some strain/parasite combinations.

ADJUVANT AND DELIVERY SYSTEMS FOR VACCINES The design of an effective malaria vaccine capable of inducing high levels of humoral and cell-mediated immunity directed at the sporozoite and exoerythrocytic stages of the parasite will require the choice of the correct T and B cell epitopes. New adjuvant and/or delivery systems must be identified that are suitable for human use, which can target the vaccine to the appropriate processing pathway for either class I– or class II–restricted responses.

The results of Phase I clinical trials of CS based vaccines for *P. falciparum*, NANP-TT (123) and R32tet32 (124), or *P. vivax*, V20NS1 (148) and VIVAX–1 (149), indicated that the immunogenicity of these vaccines was low when adjuvanted with alum. Efforts have been made since those initial trials to define new and more powerful adjuvant or delivery systems for use in humans.

The adjuvant Ribi-detox, which contains monophosphoryl lipid A (MPL), cell wall skeleton of mycobacteria and squalene, gave promising results as an adjuvant for the recombinant protein, R32-$NS1_{81}$, a fusion protein which contains 32 repeats and 81 amino acids from the N-terminus of the nonstructural protein NS1 of influenza A virus (150). The geometric

mean IFA titers in five volunteers was 1600 compared with 115 in vaccinees that received the same protein adsorbed to alum. The incorporation of the same fusion protein in combination with MPL, encapsulated into liposomes and adsorbed to alum, also induced higher titers of antibody in Phase I clinical trials (151).

Other bacterial or viral proteins have also been explored as a carrier for the NANP repeat sequence of the *P. falciparum* CS protein (152). Synthetic peptides, modified by the addition of a hydrophobic lauroyl-cysteine tail and complexed with meningococcal outer membrane proteins in proteosomes, were also effective immunogens in animal trials (153). Phase I trials of a R16HBsAg, a yeast derived fusion protein containing 16 NANP repeats and hepatitis B surface antigen (HBsAg), induced antirepeat antibodies in all 20 volunteers using smaller antigen doses than required in other vaccine trials (154). Overall, while the levels of antibody obtained with the repeat-based vaccines in the new adjuvants and/or delivery systems were higher than those obtained with alum, they did not reach the titers required for antibody-mediated protection of peptide-immunized rodents (62, 63).

CONCLUSIONS

In the last few years, much has been learned concerning the effector mechanisms of sporozoite-induced immunity, and recent findings on the T cell response of murine and human hosts have served to invalidate several previous misconceptions. A still much quoted statement about preerythrocytic malaria immunity maintains that "if only a single sporozoite escapes the neutralizing effects of antibody in the circulation of an immunized individual, this sporozoite enters the liver, develops into EEF and subsequently into blood stages, resulting in no protection." The notion that sporozoites evade the immune response by entering the hepatocyte is, of course, no longer tenable in view of the fact that the infected hepatocytes are now known to be the target of the anti-plasmodial activity of lymphokines and CTLs elicited by CS epitopes and other sporozoite antigens.

The idea that subunit vaccines are limited by parasite polymorphism must also be revised in view of data from different endemic areas, and also from mapping of T helper epitopes recognized by clones obtained from sporozoite-immunized volunteers. The data from endemic areas document the frequent recognition by lymphocytes of the same individual of multiple peptides, polymorphic as well as nonpolymorphic, within the CS protein of *P. falciparum*. These are likely to represent only a minimal number of existing epitopes, because not all activated T cells can be detected by a

proliferative response. Assays of lymphokine secretion such as IFN or IL–4, following in vitro stimulation of PBL with overlapping peptides, would probably reveal additional responders to these and other epitopes of the CS protein.

The presence of numerous T cell epitopes recognized by several inbred strains of mice has been documented in the case of the CS protein of *P. berghei*. In a recent study performed in a *P. vivax* endemic area in Colombia, similar conclusions were reached after analyzing the proliferative response of PBL stimulated with peptides from the CS protein of *P. vivax*.

The recent data do not support the pessimistic view that polymorphism of the CS protein is an insurmountable obstacle to an effective immune response. For instance, the epitope recognized by a series of CS-specific $CD4^+$ human T helper/cytotoxic clones, includes a series of polymorphic residues of the Th2R region, in addition to aa from the conserved region II. These clones recognized different variant peptides representing many of the known substitutions of the polymorphic residues. Since there are also Th epitopes in conserved regions of the CS molecule, it does not seem likely that polymorphism in a limited region of the CS protein would represent a significant obstacle to the elicitation of T help for antibody formation. The variation of the repeat domain, recently documented in *P. vivax* CS protein, however, must be taken into account because antibodies induced by one type of repeat sequence would not react with sporozoites expressing another repeat and would therefore fail to neutralize sporozoite infectivity.

Another question raised in earlier reviews was whether cytotoxic T cells would be able to target the small number of developing EEF in the infected host. The studies in the rodent model have demonstrated that both $CD4^+$ and $CD8^+$ T cell clones effectively inhibit EEF development in vivo, as well as in vitro, and protect naive recipients following passive transfer. Adhesion molecules such as CD44 appear to play a critical role in the successful targeting of the CTL to the EEF. The contact between effector and target cells is necessary for cytotoxicity and will focus the activity of inhibitory lymphokines. The participation of CTL and/or lymphokines in protection of vaccinated humans, or in individuals from endemic areas, is difficult to document. It should be pointed out, however, that human gamma IFN inhibits the development of EEF of the human malaria parasite, *P. vivax*, in chimpanzees.

The first trials of vaccines based on the repeats of the *P. falciparum* CS protein demonstrated the feasibility of conferring protection against malaria with a subunit vaccine. It also showed that, differently from what was expected on the basis of animal data, the vaccine was not sufficiently

immunogenic for humans. While less successful than expected, these trials focused research on the need to identify parasite-derived T cell epitopes and stimulated efforts to seek stronger immunopotentiators.

Subsequent data obtained in the rodent systems have shown that multiple effector mechanisms, including antibodies, helper cells, lymphokines, $CD4^+$ and $CD8^+$ CTLs, all contribute to effective protection. These protective mechanisms are evidently compensatory and redundant in the sense that high levels of either antibodies, or certain CTLs, can by themselves mediate complete protection in the immunized rodent host. There is also some indication, particularly during the early stage of the host response, that nonspecific mechanisms of resistance such as activated macrophages, NK cells, and possibly also gamma/delta cells may contribute to the protective mechanisms (34; M. Tsuji, in preparation).

There are various subjects of basic research related to the Plasmodia–host relationship in which significant advances can be expected in the next few years. One of these relates to the identification and characterization of the hepatocyte receptor(s) which mediate sporozoite adhesion and internalization into hepatocytes. Another area of interest is the characterization of the molecular mechanisms of $CD8^+$ and $CD4^+$ CTL mediated killing or inhibition of EEF development. How certain adhesion molecules contribute to the homing and localization of CTLs in the close proximity of EEF in the liver also remains to be clarified. Other promising areas for future exploration relate to the characterization of antigens shared by different developmental stages and their potential role as immunogens.

Meanwhile, to assure progress toward developing an effective malaria vaccine, human trials must be conducted. This implies the preparation by pharmaceutical companies, under FDA guidelines, of vaccine constructs safe for human use. One can only hope that consideration of the fact that hundreds of millions of individuals still suffer from malaria each year, and that millions of individuals, mainly children, die yearly of this disease, will be a sufficient incentive to maintain the effort for the development of a vaccine which appears to be within reach.

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DOMINANCE AND CRYPTICITY OF T CELL ANTIGENIC DETERMINANTS

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KEY WORDS: determinant capture, MHC-guided processing, class II MHC, antigen processing, T cell repertoire

Abstract

In this review, we first consider the inherent structural constraints for binding of a peptide to MHC class II molecules. Such parameters at the site of TCR recognition are dependent upon the efficient generation of the antigenic determinant during natural processing of the whole protein antigen. Strikingly, only a minor fraction of such potential determinants on an antigen are presented in an immunodominant manner, while the remaining peptides are silent (cryptic). Why one determinant is selected while the majority are neglected is still unresolved, but we review the experimental evidence pertaining to this choice. Thus, features of the antigen remote from the actual determinant can either steer processing toward disclosure or revelation of a determinant, or interfere with the binding of peptides to MHC (hinderotopy). The evidence is reviewed for "MHC- guided processing," where the unfolding antigen binds at an early stage to an MHC molecule through its most available and affine agretope and then is trimmed down to final size, while the rest of the molecule, including cryptic determinants, is discarded. Different MHC molecules can compete for determinants at an early stage of processing when the antigen is close to its original length. There are shifts in the hierarchy of display of dominant and cryptic determinants, and these shifts relate to

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local inflammatory states, to changes in the state or composition of the APC population, and to aspects of exogenous vs endogenous processing. The impact of this differential display of determinants on tolerance and autoimmunity is discussed.

INTRODUCTION

Recent reviews in this series have considered many aspects of the tripartite MHC-TCR-Ag recognition problem (1–8). We focus on immunodominance, the fact that a B or T lymphocyte response usually is limited to a small proportion of the potential determinants on a protein antigen. The T cell system is considered almost exclusively, and dominance is discussed along with its opposite, the notion of crypticity, which describes the failure of certain potential determinants to gain the opportunity to address T cells, for reasons summarized in Table 1. Several reviews have examined the earlier literature on immunodominance or certain other areas within our overview (9–12).

A dominant T cell determinant induces a strong T cell response upon immunization with a native antigen in adjuvant. On the other hand, a cryptic determinant makes little impact on the immune response, either to induce immunity or tolerance, unless its display is upregulated. Intermediate determinants inducing a weak response are referred to as "subdominant" under such conditions. Subdominant determinants induce a strong response when injected in the peptide form, a response which can be recalled in vitro by the peptide or the protein itself. When cryptic determinants are used as peptide immunogens, if there is only recall with the peptide, they can be considered "absolute" cryptic determinants. More interesting from the point of view of autoimmunity are the "facultative"

Table 1 Reasons for crypticity

1. Indolent processing—slow or incomplete availability of determinant during unfolding of protein molecule.
2. Excessive processing—enzymatic destruction of determinant.
3. Dominance of a flanking determinant that competes effectively for binding to the same MHC molecule.
4. Determinant capture by an unrelated MHC molecule of a more available or higher affinity flanking determinant, and subsequent destruction of the determinant in question.
5. Hinderotypic flanking region prevents access of a minimal antigenic determinant (mDET) to the MHC.
6. Hinderotypic flanking region prevents access of the MHC-peptide complex to the TCR on specific T cells.

or "latent" (12) cryptic determinants, which raise T cells that can be recalled at high doses of native antigen, or which are able to be displayed under special circumstances, respectively.

T cell "determinants" are peptides derived from a protein antigen by processing that are presented to ambient T cells by MHC class I (MHC-I) or class II molecules (MHC-II). Such determinants have agretypic amino acids that bind to the MHC molecule and epitypic TCR-binding residues. An expanded determinant-related nomenclature can be introduced here that reflects the heterogeneity of peptide length at different stages of antigen processing (Table 2).

It is profitable to consider dominance from two perspectives: (i) factors involving the determinant proper at the site of TCR recognition, and (ii) factors exerting an influence proximal to, or distal from, this site on a protein antigen. These two categories are represented in Tables 3 and 4, respectively, and in the first two major sections of this review. Under a third major heading of cellular processing mechanisms, we consider the generic attributes of a molecule that make it susceptible to the processing enzymes in its particular milieu—endosomes, endoplasmic reticulum, pep-

Table 2 A terminology for antigen processing of protein molecules

mDET =	the minimal antigenic determinant, usually defined by synthetic peptides.
DET =	mDET + FLR. The DETs are the actual determinants, most of whose residues lie within the MHC groove but include the flanking residues (FLR) that are included within the natural end products of processing.
pDET =	the prodeterminant, any one of the longer intermediate products of antigenic processing that can bind to MHC-II, and which might include several DETs.
ppDET =	the preprodeterminant, which is the first unfolded, partially or completely denatured protein antigen that could possibly bind to MHC-II.

Table 3 Factors affecting immunodominance at the TCR recognition site

1. The existence of a T cell determinant comprised of an agretope (=MHC binding site) and an epitope (=T cell receptor binding site).
2. The affinity of the agretope for the determinant binding site on the MHC molecule.
3. The density of agretopes within a limited region of the antigen.
4. The multiplicity of T cells in the T cell repertoire specific for each determinant envelope (=all unique determinants, each binding via the same agretope).
5. The affinity of these T cell receptors.
6. The relative availability of the determinant during antigen processing.
7. The number of MHC/determinant complexes presented on the surface of the APC.

Table 4 Factors affecting immunodominance distant from the site of TCR recognition

1. Uptake/endocytosis of immunogen (receptor-mediated?).
2. Existence of enzymatic processing sites permitting ready availability of agretopes.
3. Competition with an up- or downstream determinant for the same MHC molecule (=agretope competition).
4. Competition with an up- or downstream determinant for a different MHC molecule (=determinant capture).
5. Presence of a suppressor T cell directed against a non-overlapping suppressor determinant.
6. Skewed presentation to T cells by dominant B cells directed against a particular B cell determinant.

tide transport system, etc, as well as alternative cellular and subcellular pathways that might lead to variations in processing and determinant availability or display.

History of Immunodominance

The term immunodominance can be traced back to Heidelberger (cited in 13) where the reference was to B cell determinants to which a majority of a response is directed. Examples in T cell immunology in which the response was clearly limited to certain determinants, despite a potential in the repertoire for a broader response, were not published until the 1980s. In T cell proliferation studies, stringently restricted responses evidently were not limited because of the shortage of MHC binding sites or "holes in the T cell repertoire": even responses to proteins distantly related to the host demonstrated dominance. In particular, the response to hen-egg lysozyme (HEL) in H-2^a mice (A^kE^k) was limited to determinants within one cyanogen bromide fragment of HEL, despite the capacity of one of the other fragments to induce a response when it was used as immunogen (14, 15). Many instances were studied of limited responsiveness to such proteins as cytochrome c (cyt c), HEL, lambda repressor (λ rep), insulin, myoglobin (Mb), ovalbumin (OVA), and staphylococcal nuclease (Nase), (e.g. 14–29). In some reports, it was simply shown that the response was severely limited to one or two determinants (16–23), while in others, evidence was presented for "true dominance" over an unexpressed potential responsiveness to other determinants (14, 15, 24–29).

In the typical experiment, the native protein is injected in adjuvant and responses to its peptide determinants are assessed by subsequent in vitro T cell proliferation after a 10-day priming interval. Under such conditions, only a minority of the determinants, the dominant ones, induce proliferation as measured by ^{3}H-thymidine incorporation. (This format evidently doesn't permit determination of those possible determinants that

might induce not proliferation but rather T cell help; thus, the critical question about dominance among Th2 cells must be bypassed for lack of information). Furthermore, an immense difference exists between the in vivo and in vitro concentrations of antigen in these studies: In vivo, a very small amount of (originally) native antigen is released, usually from an adjuvant depot, so that the dominant determinant becomes limiting. In the typical in vitro proliferation assay, the native protein or peptide is added in the fluid phase and the small number of cells is bathed in antigen, usually at a level orders of magnitude above that achieved in the local draining node.

DOMINANCE AND CRYPTICITY RELATED TO THE SITE OF T CELL RECEPTOR RECOGNITION

The Intrinsic Structural Features of an Antigen and Immunodominance

For a determinant to be immunogenic, it should possess an optimal constellation of amino acids, complementary to the pockets in an MHC binding site groove that would ensure affine attachment to an MHC molecule and successful interaction with the TCR. Some investigators have succeeded in enhancing or decreasing the immunogenicity of an antigen by altering a small number of residues within the determinant proper.

Analysis of immunogenic peptides from several different proteins [e.g. HEL, OVA, cyt c, Mb, influenza hemagglutinin (HA), myelin basic protein (MBP) and ribonuclease (RNAse)] has revealed that individual residues in these peptides could be identified as critical MHC binding or TCR binding residues (23, 30–34). In addition, Lorenz et al (32) have demonstrated convincingly that an immunogenic peptide, RNAse 43–56, could be reconstituted by transfer of five critical RNAse residues of the peptide into an unrelated peptide backbone of the HA peptide 130–144. Similarly, Jardetzky et al (33) have shown that an HA peptide with most residues substituted to alanine, retained HLA-DR1 binding; this binding was attributable to one critical amino acid in the peptide. Wraith et al (35) have demonstrated that a T hybridoma's response to rat MBP peptide Ac 1–11, was markedly enhanced when tested with peptide Ac 1–11[4A], an enhancement attributable to its 10-fold higher relative binding affinity to I-A^u, compared to that of Ac 1–11. On the contrary, peptides Ac 1–11[3A] and Ac 1–11[6A], although, good binders to I-A^u, failed to stimulate a T cell response. Thus, positions 3 and 6 seem to be involved in interaction with the TCR. Recently, Gautam et al have shown that a polyalanine peptide with only 5 MBP residues from native Ac 1–11 is fully capable

of binding to MHC-II, of stimulating specific T cells, and of inducing autoimmune encephalomyelitis (36).

Similarly, substitution of amino acid residues in a minimal determinant (mDET) of HEL 52–61 (30), λ rep peptide 12–26 (23), or cyt c peptide 95–103 (37) can alter its capability of stimulating T cell hybridomas specific for the native peptide. This change could be owing either to processing (37) or to other causes: e.g. in the case of HEL 52–61, DYGILQINSR, conversion of 56L to 56F causes a change to nonimmunogenicity because it gains identity with a dominant peptide in mouse lysozyme to which the $H\text{–}2^k$ mouse is tolerant (54).

The above studies suggest that certain critical residues in a peptide could have a decisive influence on the immunodominance of that determinant. However, in the context of a native protein, it is important to realize that unless the appropriate pDET possessing those critical residues is generated during antigen processing, the mere presence of the same critical residues in the primary structure of the protein need not predict that the antigen fragment will be immunodominant. This is one of the difficulties with the several algorithms that have been proposed in the past to predict immunodominant sites in a protein (38–41). Two of these are briefly mentioned below.

De Lisi and Berzofsky (39) suggested that amphipathic α-helices were favored among immunodominant determinants. Algorithms based on this idea have been somewhat successful in predicting immunodominant T-helper determinants from 12 proteins. However, since immunodominant determinants of a protein such as HEL (42) span every region of the molecule in different MHC haplotypes, including diverse secondary and tertiary structures, obviously the structural features must be only one of many factors influencing the immunodominance of T cell determinants. In the HEL molecule, for example, although there were several successful algorithmic "hits," in some haplotypes the most dominant determinant was missed completely (42). Rothbard & Taylor report that a high percentage of known antigenic sites contain a 4 or 5 amino acid residue motif consisting of a charged amino acid or glycine followed by either 2 or 3 hydrophobic residues and then by a hydrophilic amino acid (41, 43, 44). This motif corresponds to one turn of an amphipathic helix. MHC-haplotype-specific motifs (45–50) have been much more successful in predicting determinants.

Affinity of MHC-Peptide Binding

CORRELATION BETWEEN AFFINITY AND IMMUNOGENICITY During processing of an antigen by antigen presenting cells (APCs), several different prodeterminant (pDET) peptides are generated, which are available for

binding to class II MHC molecules (MHC-II). Are the determinants that bind to MHC with high affinity likely to generate a better T cell response than peptides that are poor MHC binders? (It must be realized that many of these studies have actually been performed with nearly minimal synthetic peptide determinants.)

Using a panel of 12 immunogenic peptides derived from 8 different proteins and 4 different purified MHC-II molecules (A^d, E^d, A^k, and E^k), Buus et al (50) described a strong correlation between the affinity of binding and the ability of a peptide to be presented by that MHC molecule. Schaeffer et al (51) also observed such a correlation. Fourteen overlapping peptides that span the entire sequence of the protein staphylococcal nuclease (Nase) were examined for binding with the 4 MHC-II molecules above, and their T cell immunogenicity as peptides was determined. The binding capacity was graded as strong, intermediate, weak or undetectable. All of five strongly binding peptides gave a positive T cell response. However, out of 12 peptides of intermediate category, only 7 gave a positive T cell response. Finally, 36 of the 37 weak or undetectable binding events failed to give any T cell response. This failure is thought to have nothing to do with the T cell repertoire, since its acquisition is independent of foreign antigen and should be unrelated to the MHC binding affinity of the foreign determinant.

In summary, the above studies document a strong correlation between the affinity of MHC-peptide binding and the immunogenicity of the peptide. However, cryptic determinants may also bind strongly to the MHC and their crypticity may be owing to other factors. One such example, p87–96 of HEL, binds tightly to E^k (50), but is also very cryptic (see below, Crypticity Owing to Excessive Processing Within a Determinant).

PEPTIDE COMPETITION FOR ANTIGEN PRESENTATION A direct correlation exists between the capacity of a peptide to inhibit the binding of an antigen to purified MHC-II molecules and the capacity of the peptide to inhibit presentation of the antigen by fixed APCs (50, 52, 53). Peptide competition in vivo was first demonstrated by Adorini et al (54), who found that a synthetic peptide corresponding to residues 46–62 of mouse lysozyme (ML), which binds effectively to A^k, inhibited the entire priming for a T cell response when injected in molar excess into B10.A (4R) mice (A^kE°), together with HEL peptide 46–61 or native HEL. ML 46–62 was non-immunogenic in these mice. This group not only verified that the mechanisms of in vivo blockade involved direct MHC binding, it also showed that the endogenous MHC-II pathway of presentation could be inhibited, suggesting a commonality with the exogenous pathway (55, 56).

The success of in vivo competition between different peptides derived by processing of HEL seems to relate to the immunodominance of the T cell determinants (27). BIO.A (4R) mice with only a single MHC-II molecule, A^k, were immunized with HEL peptides 51–66 (dominant), 74–86 (cryptic), or 110–129 (dominant) individually, or with equimolar mixtures of peptides 51–66 and 74–86, or 51–66 and 110–129 (27). Each of these three peptides is a good immunogen alone. However, when the immunodominant peptide 51–66 was injected together with an equimolar amount of subdominant peptide 74–86, the T cell response to the latter was drastically reduced. On the other hand, injection of the two immunodominant peptides 51–66 and 110–129 together did not result in appreciable competition in vivo. Likewise, when HEL peptides 1–18/E^k (dominant) and 25–43/E^k (cryptic) were injected together in H–2^k mice, there was a marked decrease in the T cell response to 25–43, whereas the response to 1–18 was unaffected.

This limitation in competitiveness to situations in which one of the participants is weaker may be of great relevance in immunotherapeutic intervention. Several groups have successfully used this strategy to prevent the induction of experimental allergic encephalomyelitis (EAE), a T cell–mediated autoimmune disease in mice, by using inhibitor peptides that were structurally similar to the encephalitogenic peptide (35, 57) or by using unrelated peptides with good MHC binding capacity (58). What is relevant is that autoimmune disease-inducing peptides are very likely to be subdominant or cryptic (see below) and therefore may be particularly susceptible to inhibition with dominant peptides restricted to the same MHC molecule.

In vivo, inhibition need not be entirely related to affinity, and the half-life of the inhibitory peptide may be an important factor. This was the case in studying core portions of an OVA peptide from the 323–339 region that were extended with N- and C- terminal D-amino acid substitutions (53). The added stability and resistance to processing greatly increased the capacity of these derivatives to inhibit antigen presentation.

Promiscuous Binding of Peptides to MHC and its Possible Impact on Immunodominance

If a peptide can be presented by different MHC molecules, is it more likely to be immunodominant in comparison to other peptides that can only be presented in the context of one MHC allele? Several groups have reported the identification of human and mouse class II MHC restricted peptides (e.g. tetanus toxoid 830–843, hemagglutinin 307–319) that exhibit "promiscuity" (59–62) or even indiscriminate (universal) (63) binding to MHC. This promiscuity can be attributed to the look-alike quality of many

human MHC-II molecules in which the α chains share high sequence homology. Similarly, the promiscuous recognition of these peptides in association with different MHC by a single T cell clone, can be attributed to the same cause (63).

Valli et al have studied the relationship between promiscuous binding of human MBP (hMBP) peptides and immunodominance (64). In their study, 3 hMBP peptides, 13–32, 84–103 and 144–163 were found to bind to 3 or more different DR molecules. When 16 hMBP peptides were tested for their reactivity for T cell lines established from peripheral blood of multiple sclerosis patients, 3 immunodominant T cell determinants were apparent. These 3 determinants corresponded precisely to the dominant determinants within peptides 13–32, 84–103, and 144–163 which demonstrated promiscuous binding to DR molecules. These results suggest that at the population level, promiscuity can play an important role in determining immunodominance.

Berzofsky et al (65) have identified six multideterminant regions in the human immunodeficiency virus (HIV) envelope in which overlapping determinants were recognized by T cells from 4 mouse strains immunized with the HIV envelope protein gp160, and by 42 HIV-infected humans of different HLA types. It is such sets of overlapping agretopes/determinants that should maximize the opportunity for a segment of antigen to be strongly bound by a single MHC molecule, and this principle may be useful in designing peptide vaccines. Furthermore, if binding of the peptide to the same MHC groove can occur in several discrete ways, this should enhance the dominance (66).

In summary, those determinants presumably possessing attributes of a platonic ideal mDET, capable of binding to many allelic products of a single MHC locus, or to different MHC molecules in the same individual tend to be immunodominant. Similarly, those regions on an antigen with agretypic overlap, having a high density of distinct determinants, should be particularly immunodominant. Dominant ligand-MHC complexes may provide a number of distinct determinants within a determinant envelope, creating a focus available for recognition by a heterogeneous group of T cells.

Cryptocity Owing to Excessive Processing Within a Determinant

Despite demonstrable in vitro binding to MHC-II, and an available T cell repertoire, certain determinants remain cryptic. One typical example is HEL 87–96 in H-2^a mice; peptides 85–96, 81–96, and 74–96 show high affinity binding for E^k molecules, and T cell clones can be raised that are specifically activated by 87–96, but not by native HEL (129 aa residues)

or its long, partially processed derivatives (103 and 129 residues). 87–96–specific T cell hybridoma clones are also not stimulated with these long antigens, although the responses of both clones and T cells from these mice are vigorous to small peptide fragments (equal to, or less than, 23 residues = 85–96, 81–96 and 74–96), (A. Ametani, A. Sette, E. Sercarz, submitted). The crypticity of 87–96 may reflect a lack of presentation by APC of this determinant from the longer forms of the antigen, owing to "determinant capture" as described in a succeeding section ("Determinant Capture" and the Binding of Large Antigen Fragments). Accordingly, another DET such as 46–61 may represent a site at which the long fragment binds to class II molecules, and it will eventually be presented after trimming of protruding regions including 85–96.

Another possibility is that determinant 87–96 is a site degraded at an early step of antigen processing. The sequence AKKI is found between residues 95–98 of HEL. Specific proteases relating to antigen processing (67–69) can degrade peptide bonds between 2 contiguous basic amino acid residues of Lys and/or Arg indicating that K96-K97 can be targeted by such a protease. The newly produced C-terminal sequence of A95K96 then becomes a site for basic carboxypeptidases which occur extensively in the digestive as well as in the circulatory systems, cell membrane and secretory granules of mammalian cells (70). Serum carboxypeptidase N can release K96 from the C-terminal end of this newly appearing peptide. K96 was shown to be an indispensable residue for inducing T cell stimulation in the H–2^a or H–2^k haplotypes (71). These results suggest that several explanations may contribute to the crypticity of this determinant: sequential degradation by successive enzymes; the preemptive binding to MHC by some distant flanking determinant; and possibly, some steric shielding of this region from the MHC despite availability to enzymatic modification.

Impact of the T Cell Receptor Repertoire on Immunodominance

Both quantitative and qualitative explanations can be offered for how the TCR repertoire can contribute to immunodominance. First, the precursor frequency of T cell clones specific for a given determinant may be much higher than that for T cells specific for other determinants within the same protein, either inherently or owing to expansion after fortuitous antigen exposure during life. This was the explanation put forth for the marked preference for different codominant determinants among individuals of the same inbred strain, B10.A (A^kE^k), (72). Second, in case of an inherent variability in the range of affinity of TCR-peptide interaction for different determinants on the same protein, a favored site recognized mainly by high affinity TCRs might dominate the response. Adorini et al have shown that HEL peptide 25–43 binds to either I-A^k or I-E^k molecules, and it is a

much stronger immunogen in C3H (A^k, E^k) mice than in B10.A (4R) (A^k) mice (27). It was suggested that peptide 25–43 is recognized by T cells preferentially in association with I-E^k molecules, owing to a presumed low T cell precursor frequency for 25–43/A^k. However, it is also conceivable that the latter complex activates Th2 rather than Th1 cells.

Vβ gene usage is extremely restricted during the T cell response to Mb 111–121 (A^d/E^d hybrid restricted) or to MBP 1–11 (I-A^u restricted), with a single Vβ chain dominating both responses—Vβ8.2 (73–76). Interestingly, for the multideterminant region in Mb between 110–121/E^d and A^d, we already noted that a set of T cells, each with its unique Vβ, and each recognizing its own overlapping determinant, comprises the specific response (76); still another overlapping determinant has also been described in this area (77). This is just one example among others that, often, the immunodominant determinant envelope is a focus for a diversity of T cell clones (78–81). In those cases in which a T cell response is highly V_β-restricted and tolerance has been induced to that V_β or it is missing from the genome, a hole in the TCR repertoire may be demonstrated (82).

FACTORS AFFECTING DOMINANCE AND CRYPTICITY AT A DISTANCE FROM THE SITE OF TCR RECOGNITION

Distant Residues Affect Utilization of a Determinant: MHC-Guided Processing

As an initial approach in examining the effects of aa substitutions in HEL distant from particular determinant regions (83), we studied the closely related lysozyme from the ring-necked pheasant (REL) in H–2^b mice. All T cell clones specific for the multideterminant peptide 74–96 of HEL, a region with a single aa change from REL, were heteroclitic for REL, responding 100-fold better to this lysozyme. Regions near the N- and C-termini of REL were shown to have a profound effect on the handling of REL. The heteroclicity was a function of the A^b MHC-II molecule, since in H–2^d mice, clones strongly favored HEL over REL. Thus, effects related to handling of the antigen could be influential on the course and extent of subsequent T cell reactivity. In a second study, it was shown that H–2^b mice primed with HEL activated T cells specific for peptide 74–88, while priming with cyanogen-bromide fragment 13–105 only induced responses to the overlapping but distinct determinant, 81–93 (84). These results indicated that the molecular context of the determinant was paramount in determining the result of antigen processing. Sites sensitive to proteolytic enzymes may differ even among closely related antigens. Furthermore, the

context of any mDET continually changes during sequential modifications of the starting form of the antigen.

Other examples of the influence on antigen presentation of regions distant from T cell determinants were seen in the system of antigen presentation of site specifically mutated proteins. Liu et al indicated that a single amino acid substitution at Nase residue 43 outside the dominant T cell determinant regions of Nase decreased the response of E^d-restricted T cell hybridomas specific for residues 21–40 and 112–130, but not the T cell response restricted to A^d (85). In contrast, other studies in the Nase system disclosed many single site mutations not directly involved with T cell recognition that were 50–100 fold more stimulatory than the native immunogen (86). Sakurai et al also showed that the polyclonal T cell response of BALB/c mice to a recall antigen, the peptide 21–40 from bovine lactoglobulin, is different after antigenic priming with two genetic variants which had amino acid differences at residues 64 and 118 (87).

MHC-Guided Processing

Given the importance of a determinant's context for immunodominance, and the facts that distant residues on the antigen could affect the eventual specificity of the T cell response, and that this was related to particular MHC molecules, the hypothesis of "MHC-guided processing" was advanced (88, 89) as an encompassing explanation. In this view, the MHC-II molecule would encounter a tightly folded protein antigen in one of the acidic compartments of the endosomal-lysosomal system soon after preliminary processing had succeeded in partially unfolding it (ppDET), making one or a few determinants available. In the subsequent series of scissions by endo- and exopeptidases, the ppDET transforms into a series of pDETs; somewhere within this series of transformations, the occasion will arise when certain pDETs have the appropriate structure to bind strongly to MHC-II. Thus, binding to MHC-II would precede the extensive proteolytic trimming necessary before the final presentation to the T cell of the enzymatically manicured DET product in the MHC groove. The dominant determinant would be the one that initially won the competition for binding to the MHC. Provided the binding was energetically favorable and stable, the residues bound within the MHC-II groove would also be protected from further proteolysis (90, 91). This view would stress the importance of intramolecular competition between mDETs on the same long peptide for binding to an MHC molecule and likewise would introduce the idea of competition between different MHC-II molecules (e.g. A and E in the mouse) for binding to the unfolding antigen molecule. The evidence supporting this scenario is reviewed in the next section.

"Determinant Capture" and the Binding of Large Antigen Fragments

In 1984 some proteins were shown to bind without any other processing than denaturation (92), or to bind via certain locally denatured regions (93) to MHC. Later it was confirmed that whole unfolded (denatured) molecules could bind to MHC-II (94; Ametani, et al, submitted) and that three of the four antigens tested (OVA, HEL, transferrin receptor and BSA) had the revealing feature of attaching to one of the MHC-II molecules much better than to the other (94). Most pertinently, the stronger interaction always matched the dominance characteristics of the haplotype; thus, HEL bound better to E^d than A^d, and the dominant determinant in this haplotype is E^d-restricted (95). Finally, Jensen (submitted) has recently found that HEL can activate T cell hybridomas if fixed APC are pulsed with HEL in the presence of a reducing agent. Donermeyer & Allen (91) prepared a long peptide with the HEL mDET 52–61 flanked by 12-amino acid extensions with unmetabolizable D-epimers. This 34-amino acid peptide bound well, again indicating no length restrictions on MHC-II binding; furthermore, it was protected from attack at a previously enzymatically susceptible residue within the mDET. Finally, in early studies with 2 HEL-specific T hybridomas, one clone, but not the other, could be activated with full-length, reduced carboxymethylated HEL, even in the presence of chloroquine (96). This suggests in retrospect that trimming can take place outside of the acidic, endogenous processing compartments where chloroquine acts. Evidence is available that the plasma membrane itself has endopeptidases, absent from endosomes, that are active at neutral pH (97).

If a long peptide were, in fact, the entity that makes contact with MHC-II, it should be possible to show that competition takes place between the two sets of MHC-II molecules (A and E in the mouse) for initial access to the unfolding antigen molecule. We undertook the examination of several such situations and affirmed that A vs E competition for HEL determinants was evident: this competition should not have occurred if initial binding were in the form of short peptides about 15 amino acids in length (unless the peptide were promiscuous). The expected outcome was that the "winning" interaction would mark the dominant determinant; the other determinants on the dangling flanking regions would be unwilling captives which would become destroyed or cleaved off intact by a set of trimming peptidases.

A first examination of "determinant capture" compared the HEL-specific proliferative response between the B10.D2 mouse, A^dE^d, and the B10.GD, A^d, to determine the role of E^d in the response to A^d-restricted determinant in the B10.D2 (98). Indeed, both strains were HEL- respon-

sive, but in the B10.D2, the highly dominant 106–116/E^d determinant was the sole inducer of T cell activation, whereas in the B10.GD, there was a vigorous response to 11–25/A^d and lesser responses to other A^d-restricted determinants lower on the response hierarachy. The 11–25/A^d response appeared, as a much weaker response, but only at relatively large doses of HEL as immunogen in the B10.D2 or other H–2^d strains. Hence, the existence of E^d interfered with HEL-specific A^d-restricted clonal activation. This interference could be shown to not involve tolerance to E^d/peptide complexes, since the A^d-restricted TCR repertoire appeared to be intact in peptide priming experiments.

A second case was studied in which the competing determinants (91–105 and 106–116) were closer to each other than in the previous instance, and it was thought that more complete interference could be achieved, based on the idea that there would be fewer chances for dissociation between the binding determinant and the capturable determinant (H-K. Deng, L. Adorini, D. Mathis, E. E. Sercarz, ms in preparation). In the NOD mouse, 11–25/A^{nod} is dominant and 91–105/A^{nod} is subdominant. When the (NOD × BALB/c)F1 in which the E^d determinant 106–116 might have been expected to dominate the HEL-specific response was tested, the result was codominance by 11–25/A^{nod} and 106–116/E^d. The interesting feature was the complete loss of the subdominant response to the 91–105/A^{nod} determinant. Most pertinently, when CNBr-treated HEL was used as immunogen, with peptide chain disruption between met–12 and lys–13, as well as met–105 and asn–106 (but no reduction of HEL's 4 disulfide bonds), the response to 91–105/A^{nod} was reconstituted, at a higher level, equivalent to the dominant determinants! This was attributed to the prevention of determinant capture by the scission precisely between the two determinants, as well as concomitant easier accessibility to 91–105/A^{nod}. These two experimental instances of competition with up- or downstream determinants restricted to *different* MHC-II determinants are not compatible with the view that short peptides are the major initial binding substrates in the class II MHC system. Nepom has discussed competition with respect to promiscuous determinants by different MHC binding molecules: this could be considered a special case of determinant capture that would also explain protective effects in diabetes by extra MHC molecules (11).

A different argument in favor of a determinant capture mechanism comes from results in the lambda repressor system (29). In these experiments, peptide 12–26 binds better to E^d than to the A^d molecule, and yet the dominant T cells produced are A^d restricted. Naturally processed peptides derived from pDET processing of λ rep will probably experience an assortment of different flanking residues and different conformations,

whose rules of attachment to MHC-II are in the aggregate, different from those of synthetic peptides. In the sequential processing of the ppDET towards the pDET, depending on the vagaries of the everchanging flanking regions, the point will be reached where the flanking residues are, on balance, supportive of firm binding to the MHC-II molecule. During this proteolytic conversion, certain mDETs, the cryptic ones, will probably be shielded and less available than the dominant DET. Thus, binding comparisons between arbitrary synthetic peptide forms of the DET may not be relevant to the intramolecular competition between the determinants on the pDET molecule.

Another approach to examine determinant capture involves the juxtaposition of an A^u-restricted and an E^u-restricted determinant from mouse MBP to produce a chimeric peptide (CP) and to study the competition between them. Surprisingly, the entire response to the parental determinants, despite their original dominance and their obvious availability within the CP, was "subjugated" by neodeterminants of the CP (99, 100). Perkins et al (101) likewise found that λ rep 12–26/E^d was immunodominant in BALB/c mice following immunization with the λ repressor protein but gave a poor response in the context of a chimeric peptide (λrep 12–26:NP 365–80) consisting of 12–26 (E^d restricted) attached covalently to peptide 365–80 (A^d restricted) of influenza nucleoprotein.

In conclusion, immunodominance is not a function of the primary amino acid sequence alone but is influenced by the context of the determinant within the pDET or ppDET, which will be the substrate for antigen processing. There seem to be essentially different explanations for the limitation of determinants used in class I vs class II restricted responses on a protein antigen. In the former case, the binding motifs are limited, and since nonamers bind much more effectively to class I than smaller or larger peptides, the likelihood that a well-fitting nonamer will be processed from a given protein may be quite small. In any case, all the nonamers with the appropriate motif and anchor residue(s), etc, will compete for the ambient MHC-I molecules. Under these circumstances it is surprising that quite frequently, there is competition between different class I MHC molecules for available peptides, so that a single MHC type, even in an F1 heterozygote, dominates in a hierarchy, (e.g. 102, 103) probably on the basis of scarcity of peptide and affinity considerations.

Influence of the Flanking Regions of the Naturally Processed Peptides on Their Immunogenicity

Brett et al (28), in a study of crypticity of Mb determinants, postulated that flanking residues may hinder antigenic access to the MHC in certain situations in which the native molecule failed to prime for a response to a

specified peptide. They called such hindering structures "hinderotopes" (see Table 1). Experimental proof in defining the position of a hinderotopic residue was reported recently by Kim & Jang (104). A T-cell hybridoma of C57BL/6 mice responded to HEL peptide 47–61 or to native HEL at a low level: the T cell response to this peptide seemed to require an additional antigen processing step. Deletion of the single C-terminal arginine at position 61 greatly enhanced (i.e. more than 100 fold) the T cell reactivity of 47–60, and it abrogated the necessity for further antigen processing. These results clearly indicate that the presence of R61 in HEL peptide 47–61 hinders either the binding of the peptide to the I-A^b molecule or the interaction of the MHC-bound peptide to the TCR. Recent studies have shown conclusively that p46–60, but not 46–61 binds to A^b (Grewal & Moudgil, unpublished).

Bhardwaj et al (100), in the CP studies mentioned above, found that elongation of 35–47 by adding CP residues at the N-terminus had unpredicted enhancing or hindering effects on its reactivity with a 35–47 specific T cell clone. CP 9–11: 35–47 gave a better response than 35–47 itself, whereas Ac 1–11:35–47 or 5–11:35–44 provided a considerably poorer stimulus. In other clones reactive to 7–11:35–44, peptide 5–11:35–44 was absolutely nonantigenic: thus, R5 and P6 were hinderotypic.

On the other hand, Bhayani et al (105) demonstrated that the residues located in the N-terminal flanking region of pigeon cyt c peptide 95–104, outside the mDET, acted as a "stimulotope" to improve the ability of the peptides to stimulate antigen-specific T cell clones. Stimulotopes may actually provide important sites for processing. In the Nase system, both a positive and negative influence of flanking regions on the T cell response to p91–100, which represented the mDET, was observed (106). Addition of residues 86–90 made the peptide more immunogenic for Nase-specific T cell clones, whereas addition of residues 101–105 rendered the peptide nonimmunogenic. It is evident that hindering or enhancing residues external to the mDET can have profound effects on its immunogenicity. Again, the context of the mDET, even the effect of close neighbors, can have a crucial effect on immunodominance. This may particularly be the case with MHC-I–restricted determinants where the constraints of length are so influential (107).

Relationship of T Suppressor Cells and Immunodominance

Regulatory or T suppressor cells (Ts) can explain the dominance of particular determinants in some cases: the Ts act distally from the site of TCR recognition of a target determinant to ablate responsiveness to what would then become invisible determinants. In a recent review, many examples of discrete portions of well-characterized protein molecules were presented

that induced a suppressive rather than a helper or proliferative response in the T cell population (108). Such "suppressor determinants" (SD) were shown to activate $CD8^+$ T cells which could then suppress $CD4^+$ T cells directed against a distinct, helper T cell–inducing determinant (HD), but only if it were attached and proximal to the SD (109–111).

Initially, it was hard to understand how a single SD could influence the responsiveness to the whole antigen. However, in retrospect, from the vantage point of thinking about immune dominance, it is clear that the only relevant question is the relationship of the SD to the small number of dominant HD. In order to measure the activity of an SD, the readout system—the proliferation of a target Th or a target antibody producing system—must be available. In experiments in the *E. coli* β-galactosidase system, it could be demonstrated that certain potential target determinants on the whole molecule were "out of the range" of the suppressive effect of T suppressor cells (Ts) (112). The consequences of these relationships are that if the T cells are specific for a dominant determinant, and this is within the range of the suppressor T cell, no response will occur. However, if the dominant determinant is outside the sensitive range, a response will follow injection of the native antigen.

CELLULAR ANTIGEN PROCESSING MECHANISMS

Enzymatic Processing Sites Leading to Unique Naturally Processed Peptides

Some local denaturation of protein molecules is required for binding to MHC-II (90–92). Generally, proteases cannot act upon, and MHC molecules cannot bind, rigid and compact structures such as those in native globular proteins, but prefer flexible and disordered moieties in an unfolded protein. One of the candidate denaturation mechanisms is the reduction of disulfide bonds (113) which then permits at least partial unfolding of protein molecules, to allow catheptic endopeptidases to cleave key peptide bonds that open up the molecule and allow further digestion. Subsequently, the activity of an assemblage of enzymes will have profound effects on which determinant is presented as dominant. If processing is MHC-guided, ppDET → pDET protease cleavages should have strikingly different results in mice of different MHC haplotypes, at times creating highly affine determinants and in other cases destroying potential determinants.

Several proteases have been implicated as candidates for professional antigen processing, particularly for MHC-II presentation. Guagliardi et al showed that cathepsins B and D and class II molecules coexist in the

same endosomal particles in B cells (114); likewise, cathespin D is found in a membrane-associated form in macrophage endosomes (115). The involvement of these proteases was also indicated by comparing peptides that had been digested in vitro with known T cell determinants (116), and by inhibition (117) or enhancement (118) of antigen presentation with protease inhibitors. Several studies implicate other candidates: cathepsin E (119), a trypsin-like protease (120), plasma membrane-associated endopeptidases (121, 122), as well as certain specific proteases (122). Thus, a single determinant may be produced through the concerted action of several proteases, each necessary to achieve a final product. For example, a variety of protease inhibitors, assayed separately, can abrogate a response to a single determinant (123). Likewise, cathepsin D can be responsible for the initial cleavage of myoglobin, while cathepsin B is inactive as an endopeptidase but does catalyze the C-terminal trimming of the released fragments. This sequential activity can account for all major determinants in the H–2^d haplotype (116). These authors point out that the motif necessary for the cleavage by a particular enzyme may correlate closely with the recognition motif of one MHC-II molecule and not another. This may represent an example of convergent evolution as well as a mechanism through which processing and MHC recognition may be intimately linked.

However, for different antigens, or in particular APC, an entirely different degradative scheme could occur. In certain cases, a crucial first step involves a single cathepsin, which then permits the processing of diverse determinants on a molecule (117): this is consistent with the idea that the molecule just needs to be opened up to allow further digestion by other enzymes. In A20 (H–2^d) B lymphoma APC, as well as in most resting and activated B and T cells, but not peritoneal macrophages, cathepsin E is necessary for OVA processing (119). This aspartic proteinase is not a housekeeping, lysosomal hydrolase, but rather, it represents one instance of heterogeneity in antigen-processing systems in different locales.

One reasonable conclusion from all these studies is that determinants emerging from native protein digestion may have experienced a series of protease cleavages which could differ among particular cells. The sites of cleavage may have special relationships to certain MHC molecule per se as well as to the variable lifetime of processing intermediates in different subcellular compartments (124). The natural products of antigen processing are derived via a continuum from the ppDET through the pDET to the final DET found within the MHC groove. As mentioned elsewhere in this review, the molecular context of the mDET will progressively change during this conversion and could be the decisive factor in establishing the position and the moment of MHC contact.

Efficient Endocytosis: The Example of B Cell Processing and T-B Reciprocity

Antigen presentation is also related to the efficiency of internalization and denaturation. Specific antibody receptors on antigen-presenting B cells play an important role in greatly increasing the uptake of specific antigen (about 10^4-fold) (125–127). Guagliardi et al (114) indicated that antigen internalized into antigen presenting B cells is transferred together with surface Ig into early endocytic compartments containing proteases and MHC class II molecules. Since these molecules are co-localized in the same organelles, not only the antigen itself but also the antigen bound to its specific antibody can be transferred to a binding cleft in an MHC molecule, leading to qualitative effects on final processing.

In older work, spatial relationships between a T cell and a B cell determinant on an antigen were shown to influence T cell–dependent B cell activation and differentiation to antibody-producing cells, i.e. a steering mechanism (128, 129). When the nature of T-B collaboration became clearer the reciprocity of the relationship was highlighted (130). It was demonstrated that B cell receptor specificity circumscribes the specificities of T cells that can be activated during antigen presentation (131, 132), in this sense skewing the resulting immunodominance or crypticity of the T cell population. In the case of the multidetermined protein antigen, previously primed T cells, specific for a single T cell determinant, could preferentially activate B cells specific for a particular subset of B cell determinants on a protein antigen (133; M. Palmer, E. Sercarz, manuscript in preparation); in some cases, intermolecular help could be provided to a variety of T cells (134). Apparently, in this and other examples of directional help (135) and T-B reciprocity (130), effects are not absolute, but could be influential in vaccine design. Thus, the choice of which T cell will become dominant should depend to some extent on the antigen processing pattern occurring in specific B cell APC, which indeed differs in B cells specific for unique determinants on the same antigen (136). An influence of paratope specificity of surface immunoglobulin upon antigen processing patterns was also found in antigen presentation by macrophages of antigen-antibody complexes (132).

The question of whether cells specific for overlapping T and B cell determinants can collaborate is still open. Thomas and his co-workers (137, 138) found extensive commonality of the T and B cell determinants within influenza hemagglutinin. On the other hand, Ozaki & Berzofsky (139) showed that B cells present overlapping T cell determinants less well than if there were no overlap. In other studies, when a T cell determinant overlaps fully with a B cell determinant, its T cells cannot help in antibody

production by specific B cells (140). These paradoxical findings could relate to the degree of "protection" afforded to the antigenic determinant by Ig receptors of differing affinity for residual, partially processed fragments of the antigen.

Changing the Level of Antigen Presentation

ACTIVATION OF APC AND INCREASED DISPLAY OF CRYPTIC DETERMINANTS Several groups have demonstrated that activation of APC can render determinant display more efficient. In C5 deficient mice, APC do not synthesize sufficient pro-C5 to activate C5 specific T cells unless the APC are stimulated with IFNγ (141). IFNγ-induced stimulation of macrophages results in upregulation of aspartyl protease activity (142; J. Blum, unpublished) suggesting that different proteases dominate processing of antigen in resting vs activated cells. Changes in the proteasome system also are regulated by interferon (143, 144) and can lead to alterations in processing in inflammatory states. Another result of inflammation, heat shock, has been reported to increase presentation of endogenous antigen (OVA) on MHC-II by OVA-transfected B lymphoblastoid cells (145).

Lin et al have shown (146) that in the mouse, activation of autoantigen specific B cells by cross-reactive, xenogeneic human cytochrome C (cyt c) is required for rendering previously cryptic determinants on autologous cyt c immunogenic. In this model, self tolerance is subverted in three sequential steps. First, T cells are primed to "foreign" determinants on human cyt c. Second, B cells that are specific for shared determinants between human and murine cyt c (autoreactive B cells) capture and present human cyt c to T cells recognizing "foreign" determinants on cyt c; this interaction leads to activation of the B cell. Subsequently, these activated, cross-reactive B cells are able to present autologous murine cyt c in a more efficient manner which leads to a second wave of priming to previously cryptic self determinants on the autoantigen, mouse cyt c. Thus, the especially efficient presentation of both foreign and self determinants on the molecule may be one mechanism of "breaking" self-tolerance, although in fact, tolerance had never been induced to the cryptic determinants.

ALTERNATIVE PROCESSING IN CELLS OF DIFFERENT LINEAGE OR IN DIFFERENT ORGANS APC of different cell lineages can process differentially as evidenced by Moreno et al (147) who noted that class II positive fibroblasts, transfected with HEL, presented a determinant of HEL which B cells, transfected in a similar manner, did not present. Why this happens has not been studied sufficiently as yet, but differences in invariant chain

expression (148) and in endosomal-lysosomal proteolytic activity could account for this finding. Similarly, with different proteins being expressed in different cell lines, the spectrum of self peptides competing for MHC binding will differ among cell lines, resulting in differential display of nominal self antigens. Lipham et al have shown (149) that the interphotoreceptor retinoid binding protein (IRBP), involved in autoimmune inflammation of the retina, contains two contiguous determinants, the immunodominant peptide 1179–1191 and the cryptic peptide 1158–1180. They proposed that this cryptic determinant is not produced intracellularly by APC, but that it could be generated locally via extracellular cleavage of IRBP by retinal proteases in vivo. They showed that digestion of IRBP by endoproteinases yields peptide fragments that, unlike native IRBP, are capable of stimulating T lymphocytes specific for the cryptic IRBP peptide, 1158–1180.

ENDOGENOUS SYNTHESIS VS EXOGENOUS UPTAKE OF ANTIGEN Endogenously synthesized antigen is very efficiently presented on MHC-II (150–152), contrary to previous opinion. The nature of this pathway is currently under study. In addition to a more efficient delivery to the acidic endosomal compartment, another possibility is that a set of special proteasomes would process endogenous antigen for MHC-II. Furthermore, special peptide transporters seem to be required for loading of cytosolic peptides onto MHC-II (153, 154).

Using the standard lymph node proliferation assay Yamashita & Heber-Katz (155) noted dominance of a single antigenic determinant (residues 241–260) in the response of H–2^d mice to glycoprotein D (gD) of herpes simplex virus (HSV). However, when mice were primed with live HSV the pattern of responsiveness was radically different in the draining lymph node population: 17 peptides of gD were found to behave in a codominant fashion. It appears that this can be attributed to the efficient endogenous synthesis of antigen by infected APC as opposed to exogenous uptake of antigen by noninfected APC. Similarly, Eisenlohr & Hackett (156) using an influenza neuraminidase (NA) specific T cell hybridoma, detected I-E^d restricted presentation of a NA determinant on infected APC, but not on APC which had been pulsed exogenously with the noninfectious antigen. Variations in determinant display among different types of APC were reported by Moreno et al (147). While determinant 25–43 of hen egg lysozyme (HEL) was found to be presented efficiently to a HEL specific T cell hybridoma by B cells, both after uptake of exogenous HEL or after endogenous synthesis by HEL-transfected B cells, an Ia^+ fibroblast APC line was only capable of generating the determinant

when transfected with HEL, but not after endocytosis of this antigen.

As with infectious antigens, dominance of several determinants on the autoantigen MBP was detected in chronic EAE (157–160). The repertoire primed by endogenous MBP exhibited a broader fine specificity than that after immunization with MBP/CFA suggesting that these alternative pathways of processing have relevance for autoimmunity.

DEVELOPMENT OF DOMINANCE WITHIN THE T CELL RESPONSE

The Dominance Pattern of the T Cell Response to a Foreign Antigen Does Not Change During the Course of Immunization

Dominance of determinants on protein antigens has been generally related to display of determinants in the regional lymphoid tissue 1–2 weeks following primary exposure to antigen in adjuvant. Ordinarily, long-term dominance has not been studied because the local response in the draining lymph node is transient and usually vanishes by one month after immunization. However, in the splenocyte population, antigen-induced recall responses by primed $CD4^+$ cells continue for at least 6 months after immunization (157, 161). Importantly, the pattern of dominance and cryptticity was found to be identical in the lymph nodes and spleens of individual mice from several H–2 haplotypes when compared 9–10 days after immunization (157).

To study whether changes in the expressed T cell repertoire occur with time, long-term response to HEL in BALB/c and (SJL × B10.PL)F1 mice was followed. The typical dominance of 106–116/E^d was maintained even at 6 months in the absence of "promotion" of cryptic determinants. Interestingly, the lymph node response to HEL in (SJL × B10.PL)F1 mice is also characterized by single determinant dominance by 30–53/A^u, while peptides 11–25/A^s and 116–129/A^s behave in a cryptic fashion (157). In the SJL parents, the latter determinants are immunodominant, and they also are immunogenic in F1 mice when injected as peptides (P.V. Lehmann, unpublished). In the F1 mouse, the late spleen retains the early lymph node constellation of specificity (157). Therefore, in two systems, where there was a clear potential for recruiting weak determinants into the response, this did not happen; i.e. changes in the APC compartment which might have rendered presentation of subdominant or cryptic determinants more efficient were not in evidence.

To test the converse possibility, that a primary multideterminant T cell response would focus down to one dominant peptide with time, the HEL response in B10.A(4R) mice was followed. In B10.A(4R) mice, individuals

respond to four codominant determinants in the classical lymph node assay, namely peptides 20–35, 30–53, 46–61, and 116–129, but the hierarchy of determinants within individual mice shows considerable inter-individual variation (163). No changes in the fine specificity or the dominance hierarchy of the HEL response were found in the spleens of individual mice when tested 2 weeks and 2 months after immunization (P. V. Lehmann, T. Forsthuber, A. Miller, E. E. Sercarz, ms.). Since T memory cells appear to be short-lived and T cell memory seems to depend on antigen (164), a reduced display of determinant might have been expected to narrow the primed T cell repertoire. However, it appears that once established, the pattern of dominance in the T cell response does not change after immunization with the "foreign" antigen, HEL.

The T Cell Repertoire Spreads To Cryptic Determinants in Response To a "Self" Antigen

The results with HEL are in striking contrast to the development of dominance in the T cell response to the "self-antigen" myelin basic protein (MBP) (157). Nine days after immunization with MBP, the primed repertoire was found to be confined to Ac1–11, both in draining lymph nodes and in spleen cells of (SJL × B10.PL)F1 mice. However, 21–180 days after immunization, several previously cryptic determinants of MBP recalled proliferative responses in the spleen, namely those within peptides 35–47, 81–100, and 121–140, a diversification which also has been noted in other studies (157–160).

Most importantly, reactivity to the latter determinants was also detected after induction of EAE with MBP peptide Ac1–11 alone! (157) This demonstrated that additional peptides of endogenous MBP became immunogenic during Ac1–11-induced encephalomyelitis. The determinants of MBP which behave cryptically following primary immunization in the lymph node (35–47, 81–100, and 121–140) appear to be displayed in a dominant fashion in the CNS, since immunization with these peptides induces EAE (development of the disease is dependent on recognition of the determinant in the target organ). It is of interest that in multiple sclerosis, a broad reactivity to a variety of MBP peptides has been noted (165–168). It can be assumed that at the onset of the disease, a more limited reactivity would have been seen, but this may be difficult to prove in the human context. Apparently, the presentation of MBP is different for exogenous antigen in the lymph node than for endogenous protein in the CNS. In summary, the cryptic self can be thought to undergo waves of display throughout life; during these episodes, T cell memory directed towards the cryptic determinants may be expanded, giving rise to autoaggression.

IMMUNODOMINANCE OF SELF-PEPTIDES AND AUTOIMMUNITY

Tolerogenic and Nontolerogenic Self-Determinants

Immature T cells that recognize (self) antigen in the thymus are deleted from the maturing T cell pool or inactivated (169–172). Also, mature T cells can be rendered tolerant by antigen recognition under certain circumstances (173). Both pathways of tolerance induction are dependent on recognition of determinants of processed antigen. From experiments described below, it can be concluded that tolerance will involve only those determinants on an autoantigen that are efficiently displayed to the T cell pool (dominant self), sparing poorly presented determinants on the molecule (cryptic self).

Adult mice, rendered tolerant to HEL by intravenous injection of the protein, do not respond to a subsequent challenge with the whole protein or peptides encompassing dominant determinants. However, such mice mount T cell responses to cryptic determinants (173). Supporting this view, Gefter et al have shown that adult tolerance induction to the chimeric peptide formed by two peptides results in abolition of only the T cell response against the dominant determinant (174). The same constellation has recently also been observed during spontaneous development of tolerance to endogenous antigen. Thus, H–2^d mice transgenic for HEL, were nonresponsive to its single, dominant E^d-restricted determinant but did respond to cryptic determinants on HEL (175). Apparently, the relative hierarchy of dominance established in the immune periphery (lymph node) after immunization with CFA also holds true for the thymus; presentation of self-determinants during negative selection did not occur at sufficiently high efficiency to inactivate autoreactive T cells specific for cryptic determinants.

Self-MHC molecules can be considered as prototype autoantigens which are displayed as processed peptides to T cells during tolerance induction. In a model in which both class II and class I MHC peptides were employed as immunogens, cryptic determinants that evidently could elicit a proliferative response were present in each instance, demonstrating that the entire self-MHC-reactive T cell repertoire was not purged (176). Similar findings were made by other investigators (177–178). Consider the self-MHC determinants encompassing the polymorphic α helices of D^d and L^d molecules. Two peptides, L^d 61–85 and D^d 61–85, were shown to bind with high affinity to the class II restriction elements of B10.A mice (A^k, E^k, L^d, D^d) but only L^d 61–85 was capable of inducing a T cell response upon immunization (176). The failure of D^d 61–85 to stimulate a T cell response

is presumed to be a consequence of tolerance induction since there is an immune response to D^d 61–85 in B10.BR and CBA mice which share MHC-II (A^k, E^k) with the B10.A strain but express D^k. Immunogenicity of L^d 61–85 in B10.A mice, in spite of its high affinity for self MHC-II, probably reflects the lower level of expression of L^d molecules as compared to D^d (179, 180). The L^d peptide is capable of inducing tolerance, but only T cells directed against the dominant determinant, 65–80, are affected.

Lin & Stockinger have shown (181) that mice deficient for the fifth component of complement (C5), unlike normal mice, do not possess the secreted form of C5 and can be readily immunized to serum-derived, murine C5. Although APC from C5-deficient mice do not secrete C5, they synthesize the precursor protein (pro-C5) and stimulate class-II–restricted, C5-specific T cell clones without addition of exogenous C5 (141). Immunization of the deficient mice with pro-C5 induces normal C5-specific T cell proliferation. In this case, it is likely that the failure of pro-C5 to induce T cell tolerance can be attributed to its ten-fold lower level of synthesis by macrophages in C5-deficient mice as compared to normal macrophages. Collectively, these data strongly suggest that self-tolerance is only induced to efficiently presented, dominant determinants, but not to weakly presented, cryptic, determinants on autoantigens.

Propagation of Autoimmune Disease

Evidence that determinant dominance is not a fixed feature of antigen presentation was presented in a previous section (Cellular Antigen Processing Mechanisms). Factors affecting the display of a determinant as dominant or cryptic include cell lineage, the activation state of the APC, as well as the intracellular vs extracellular origin of the autoantigen. Therefore, if any combination of circumstances disrupts the hierarchy in the presentation of self-determinants such that cryptic determinants become displayed in a dominant fashion, the immune system will respond to the newly emerging "self" as if it were "foreign." Determinants presented cryptically during the establishment of tolerance in the thymus and in secondary lymphoid tissues, may be displayed dominantly under certain circumstances and especially during chronic, T cell–induced, lymphokine-mediated inflammation. This will lead to recruitment of bystander, naive T cells, some of which are specific for cryptic determinants. Additionally, these lymphokines, can induce expression of MHC-II on cells that constitutively are MHC-II negative (182) and also upregulate accessory molecules that function as costimulatory signals for T cell activation. Accordingly, additional antigenic determinants become displayed, and importantly, all the requirements for priming a T cell response are also

generated. Autoimmunity directed against one autoantigen (MBP) can spread to involve another (phospholipid protein) (183). The auto-aggression will probably remain confined to the local area of inflammation where cryptic determinant display is upregulated.

Cryptic Self-determinants Shape the Repertoire for the Dominant Determinants of the Homologous Foreign Protein

Priming and repeated exposure to cryptic self will create and expand specific T cell memory, which should heighten susceptibility to auto-immune disease. For example, following exposure to an infectious agent expressing a dominant determinant cross-reactive with a cryptic self-determinant ("molecular mimicry"), the memory T cell repertoire will be readily recruited to local sites of upregulated antigen presentation in a target organ.

At present, it is believed that foreign homologous antigens induce responses largely dependent on their degree of difference from the host antigen. However, what may be most important is the relationship between dominance and crypticity in the foreign antigen and its self-homolog. High crypticity of a self-determinant guarantees the existence of T cells directed against it. Even a single difference in a flanking residue essential for processing may lead to a dominant response to HEL, whereas in mouse lysozyme (ML) the identical determinant could be cryptic.

To test these ideas, the ML peptide-specific T cell repertoire has been studied in mice of different MHC-II haplotypes. The pattern of reactivity to ML peptides, defining the cryptic T cell repertoire, was unique in mice of different haplotypes, indicating that crypticity is MHC-associated and not a structural attribute of the determinant (184). On comparison of the pattern of response of mice of H–2^k, H–2^b and H–2^d haplotypes to the panel of ML and HEL peptides, a striking positive correlation was evident between the patterns of response to cryptic ML peptides and to dominant HEL determinants in each strain. Despite the vast difference (55 a.a.) between ML and HEL, the dominant HEL-specific repertoire corresponds almost precisely to the cryptic self-repertoire to ML! (K. Moudgil, E. Sercarz, manuscript in preparation). Therefore, it appears that the T cell repertoire to a foreign protein is shaped and constrained by the cryptic determinants of the homologous self-protein.

POSTSCRIPTS

The Homunculus and Immunodominance

Immunodominance is a pervasive motif throughout immunology: the ability to regulate responsiveness is a key issue in the development of the

immune system, and a high premium must have been paid during evolution for cogent, focused regulatory mechanisms. MHC-guided processing represents one example of a device for ensuring dominance and reducing complexity. Thus, whether studying the limited number of cytotoxic T cell determinants, or predominant idiotypes, or clonal dominance, or MHC-II antigen presentation, the production of too many competing entities might preclude effective regulation (185). Irun Cohen likened this to the depiction of the neurological homunculus (186), in which organs receiving heightened innervation are overrepresented. In the immunologic version, he postulates that certain self-determinants (and self-antigens) as well as certain idiotopes are given regulatory roles that lead to their predominance in immune arenas. The organism either develops a suitable regulatory circuitry during ontogeny to constrain these favored, homuncular motifs, or they are built into the system ab initio. Looked at from the point of view of TCR which are involved in such regulatory networks, $V_\beta 8$ reportedly is used in far excess in rat EAE as well as other autoimmune diseases (187); this may be one approach to limiting the protagonists and centralizing regulation.

Aleatory Intramolecular Competition

We have seen how dominance and crypticity can be considered empirical, aleatory phenomena, at least from the viewpoint of a single determinant and its hierarchical position in a particular MHC haplotype. From any perspective, whether a particular "index" determinant in a single molecule will be dominant depends in large part upon its affinity for the ambient MHC molecules. However, overriding factors will be unrelated to the intrinsic affinity of the index mDET and will involve the intricate connections between processing and availability of determinants and the intramolecular competition that should be a major factor in decisions about dominance: (i) whether that part of the antigen has a high relative availability will depend on processing patterns that relate to residues in distant flanking regions, as well as hinderotypic/stimulotypic residues in nearby flanking regions; (ii) the relative affinity vis-à-vis up and downstream determinants on the antigen that will compete for attachment to the same or different MHC-II molecules. Under the usual conditions of antigen shortage in the acidic endocytic compartments, these are the crucial events in defining the immunodominant determinant. From the vantage point of the index mDET, its position in a dominance hierarchy is likely to be less an intrinsic feature of the mDET than an aleatory outcome of exactly which type of DETs live in the neighborhood. Once this is established, and the investigator studies one molecule in syngeneic individuals, prediction of dominant response patterns becomes easier.

Concluding Remarks

We have pointed out that with self-molecules, these patterns can be very dynamic, especially when cryptic self-determinants have an opportunity to surmount their display threshold and come out of hiding. Although they may later return to obscurity, local inflammatory events may draw them out yet again. The result should be an episodic stimulation of memory within the self-reactive repertoire, making autoimmune aggression an increasingly more likely event with age. Additionally, as we have noted, the repertoire directed against cryptic self molecules acts to focus the response against related foreign molecules.

With respect to vaccination, perhaps the single most useful hint would be to separate different determinants in the immunogen and not attempt to link them into one massive entity, so that intramolecular determinant capture can be avoided. It may be of value to seek algorithms characterizing the most promiscuous determinants. Eventually, it should be possible to prepare multideterminant arrays representing repeats or overlaps of dominant, promiscuous determinants.

We have tried to bring to the forefront accumulating ideas about the possible succession of events in the sequential processing of compact protein molecules. The chief realities—of dominance of a limited number of determinants, and of crypticity within every antigenic molecule—argues for a mode of antigen handling that would explain these phenotypes. A new emphasis has been placed on regarding the protein antigen molecule as an everchanging entity during its early unfolding, with each microprocessing event altering the relationship among determinants and their exposure to the proteolytic machinery as well as to MHC-II active sites: a point is reached at which the prodeterminant complex can bind strongly to the MHC at the dominant site. This view places processing events in a decisive position with regard to determinant choice but not to the exclusion of inherent structural constraints. It can be postulated that with regard to dominance, intramolecular competition is likely to be more important for MHC-II and intermolecular competition for MHC-I: the former provides a basis for rationalizing competition between different MHC allelic products.

Many questions remain, but the rapid accumulation of knowledge about antigen processing and presentation as well as MHC structure/function promises ultimately to clarify the nature of determinant hierarchies. Yet, ". . . nature is never spent; There lives the dearest freshness deep down things (188)" as well as daunting complexity.

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ENDOTHELIAL-LEUKOCYTE ADHESION MOLECULES

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Abstract

One decade ago, vascular endothelium was commonly considered a "non-stick" lining of blood vessels that functioned only to prevent blood coagulation and to separate the vascular space from tissues. By comparison to many other cell types, endothelial cells were thought to be less active, less complex, and less interesting. Since that time, research concerning the endothelium has expanded dramatically and produced a new image of the vascular lining as an active participant in a wide variety of pathophysiological processes, including inflammation and immunity. Nowhere has the excitement been more intense than in the study of the molecular mechanisms of leukocyte adhesion to endothelium. Recent efforts resulted in the identification, characterization, and cloning of multiple endothelial cell–surface glycoproteins that support adhesion through an interaction with specific ligands (or counter-receptors) on leukocytes. The selectins, two of which are found on endothelium and one on leukocytes, support adhesion through the recognition of carbohydrates. Endothelial members of the immunoglobulin superfamily including ICAM–1 and VCAM–1/INCAM–110 bind to leukocyte cell-surface integrins. In various combinations, these and other molecules support leukocyte adhesion to the vessel wall and extravasation, key steps in our response to infection and tissue injury.

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INTRODUCTION

Focal leukocyte extravasation is a hallmark of inflammation (1). Typically, it is heralded by a local vasodilation that increases blood flow and delivery of leukocytes, and is accompanied by an accumulation of fluid and plasma proteins in the involved tissues. Endothelial cells participate in these events in several distinct ways (reviewed in 2, 3). For example, they produce vasoactive compounds including prostacyclin (PGI2) and endothelial derived relaxing factor (EDRF, nitric oxide) which cause smooth muscle relaxation. In addition, endothelial cells can make cytokines such as interleukin–8 (IL–8) and lipid-based mediators like platelet activating factor (PAF) that stimulate leukocyte functions, including adhesion. Finally, endothelial cells constitute a major barrier against fluid loss from vessels, and it is their retraction and/or damage that results in the increased permeability at sites of inflammation.

Central to the processes of inflammation is a dramatic increase in endothelial cell surface expression of molecules that support the adhesion of blood leukocytes. In response to certain mediators like thrombin and histamine, endothelial cells redistribute adhesion molecules from storage granules to the surface within minutes; in response to endotoxin, IL–1, or TNF, endothelial cells biosynthesize and express at least three adhesion molecules over a period of hours to days. When studied in vitro, the individual endothelial contributions to inflammation seem almost unconnected; some are immediate and transient, others delayed and prolonged. Together in the body, however, they provide for an elegant orchestration of cellular and fluid movements designed to neutralize and eliminate foreign substances. Unfortunately, in certain settings, control mechanisms appear to fail, and the inflammatory process becomes extreme (4). The ensuing tissue damage contributes to important human diseases.

A rapidly expanding body of data has revealed the structure, function, and expression of endothelial cell surface molecules that support the adhesion and extravasation of leukocytes. In this review, I recount the characterization of these molecules, and attempt to provide insights into their biological and medical significance. The first sections emphasize studies performed in vitro and are organized according to receptor/counter-receptor pairs, including selectin-carbohydrate interactions and immunoglobulin-like cell adhesion molecule (IgCAM)-integrin interactions (Figure 1). In most cases, emphasis is placed on the description of the endothelial cell molecules. Subsequent sections focus on the expression and function of endothelial-leukocyte adhesion molecules in vivo and their involvement in human disease. I apologize for any deficiencies or

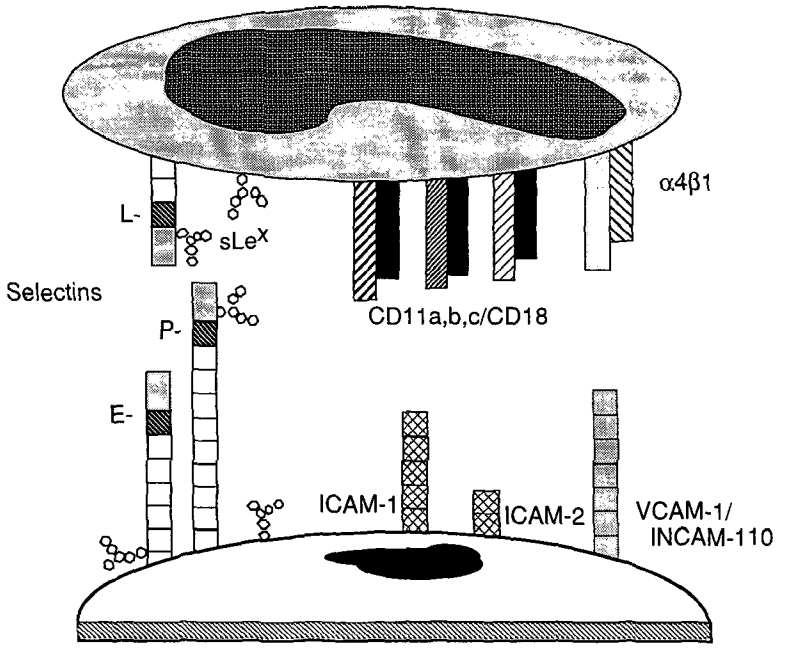

Figure 1 Endothelial-leukocyte adhesion molecules. The adhesion of leukocytes to endothelial cells is mediated by specific interactions of receptor-ligand (counter-receptor) pairs. These include the selectin-carbohydrate interactions and IgCAM-integrin interactions shown here. Monocytes contain a full array of adhesion molecules, whereas other leukocyte types express some but not others. For example, neutrophils do not express the $\alpha 4\beta 1$ integrin which binds to endothelial VCAM–1/INCAM–110. Most lymphocytes do not express CD11b/CD18 or CD11c/CD18. Neutrophils, monocytes, and a subpopulation of T lymphocytes express the carbohydrate sLe^x or other carbohydrate ligands for E- and P-selectin. Other endothelial and leukocyte cell surface molecules also participate in cell-cell interactions (see text).

oversights in this review; other recent discussions on adhesion molecules may also be of interest (5–12).

SELECTIN-CARBOHYDRATE INTERACTIONS

A new family of adhesion molecules, now called selectins, was first recognized in March of 1989 when published articles presented the cDNA sequences of three cell surface glycoproteins found on endothelium, platelets, and leukocytes (13–16). Two of these molecules, endothelial-leukocyte

adhesion molecule 1 (ELAM–1) and the murine lymph node homing receptor or MEL–14 antigen (Table 1; see 17 for nomenclature review), were known to be expressed by cytokine-activated endothelial cells and leukocytes, respectively; each was thought to participate in endothelial-leukocyte adhesive events in one or more settings (see below). The third molecule, known as platelet activation dependent granule-external membrane protein (PADGEM) or granule membrane protein 140 (GMP–140), was first identified on platelets activated by thrombin (18, 19); subsequent studies demonstrated its expression on thrombin-stimulated endothelial cells (20, 21). Sequence data for each of the three molecules predicted a Type I transmembrane protein with an N-terminal lectin-like domain, an epidermal growth factor (EGF) repeat, and a variable number of modules ($\sim$60 amino acids each) similar to those found in certain complement binding proteins (Figure 2). The term selectin was proposed (22) to highlight the amino terminal lectin domain and to indicate the selective function and expression of these molecules. A standard nomenclature has been agreed upon (17) which designates each family member according to the cell type on which it was originally identified: E-selectin (endothelium), P-selectin (platelets), and L-selectin (lymphocytes) (Table 1). Since the realization that these three molecules contain an amino terminal region related to the carbohydrate recognition domains of calcium-dependent (C-type) animal lectins (23), an intensive search for carbohydrate ligands has developed (12). The result has been a dramatic convergence of the fields of cell-cell adhesion and carbohydrate biochemistry.

E-Selectin

In 1985, it was demonstrated that the cytokines interleukin–1 (IL–1) and tumor necrosis factor (TNF), as well as bacterial endotoxin, could act on endothelial cells to increase dramatically the adhesion of isolated blood neutrophils (24, 25). E-selectin (originally ELAM–1) was identified as a participant in this process, using monoclonal antibodies generated against cytokine-activated endothelial cells (12, 26–28). The characterization of E-selectin function was extended by the demonstration that a recombinant form of this molecule could support neutrophil adhesion (13). Subsequent studies in vitro have suggested that E-selectin can also participate in the adhesion of monocytes and a subpopulation of memory T-lymphocytes to cytokine-activated endothelial cells (29–33). Eosinophils and basophils may also bind to E-selectin (34, 35).

E-selectin expression is largely restricted to activated endothelial cells. Cultured endothelial monolayers synthesize and express E-selectin after exposure to endotoxin, IL–1, or TNF (13, 28). Maximal surface expression is observed at 4 to 6 hr, followed by a decline toward basal levels by 24–

Table 1 The selectin family of adhesion molecules: expression and function

	Old names	Expressed by	Regulation	Binds to
E-selectin	ELAM-1	endothelium	IL-1, TNF, LPS inducible expression (hours) RNA, protein synthesis	neutrophils monocytes some T cells
P-selectin (CD61)	PADGEM GMP-140	platelets endothelium	thrombin, histamine, others from storage granules (minutes) cytokine inducible (hours)?	neutrophils monocytes some T cells
L-selectin	mLHR, Leu8 TQ-1, gp90^{MEL} Lam-1, Lecam-1	lymphocytes monocytes neutrophils	constitutive surface expression modulation of activity shed after cellular activation	lymph node HEV activated endothelium

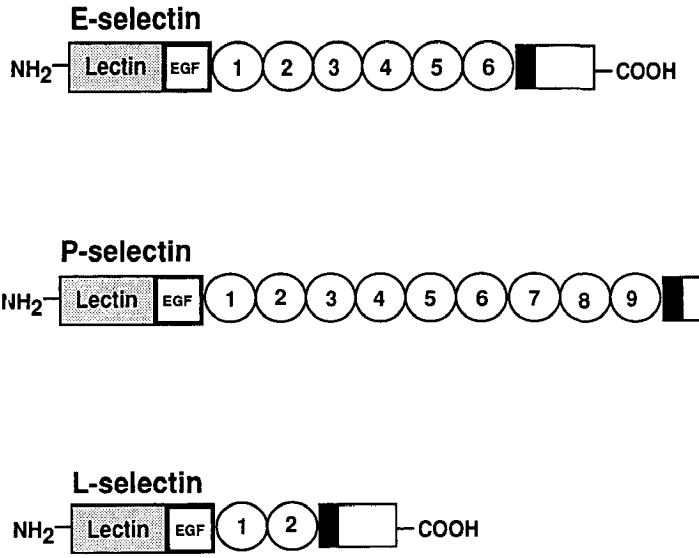

Figure 2 Schematic structures of E-, P-, and L-selectin depict amino terminal lectin domain, EGF repeat, discrete number of complement regulatory-like modules, transmembrane domain, and cytoplasmic tail.

48 hr (Figure 3). Interferon-γ alone does not induce the expression of E-selectin, but it can prolong its expression in response to TNF (36, 37).

Two key characteristics of E-selectin expression, cytokine inducibility and tissue specificity, have prompted substantial interest in the regulatory elements of its gene. The human E-selectin gene contains sequences consistent with NF-κB and AP–1 binding sites (38–40). The NF-κB site may be necessary but not sufficient for cytokine-inducible expression of the human gene (39, 40). Interestingly, the 5′ flanking region of the murine E-selectin gene can also support cytokine-inducible molecular expression but does not contain a sequence corresponding to the consensus NF-κB site (41). Further investigation of E-selectin expression could yield important insights into the regulation of inflammation and suggest ways to control it. Reagents capable of blocking E-selectin expression could prove to diminish inflammatory responses in vivo. Interestingly, a recent study suggests that the anti-inflammatory effects of corticosteroids involve such a mechanism (42).

P-Selectin

P-selectin (CD–62) is a transmembrane glycoprotein of approximately 140 kDa that is associated with α- and dense-granules in resting platelets (18,

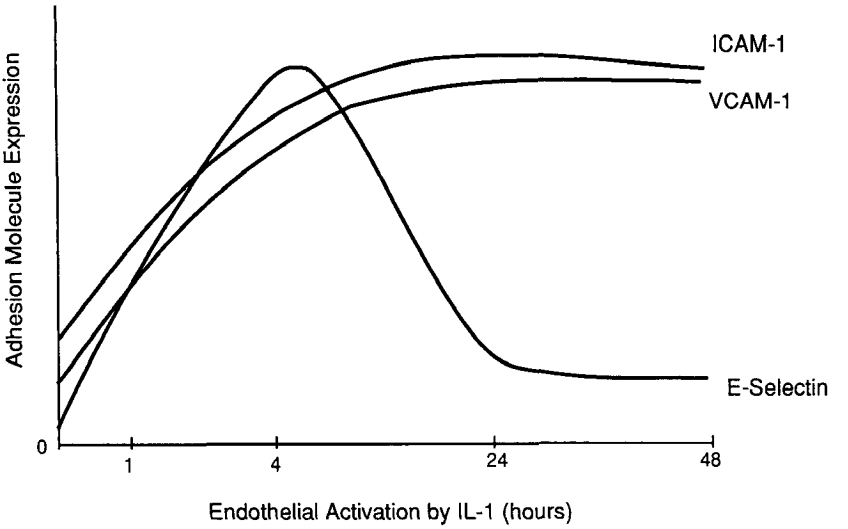

Figure 3 Time-dependent expression of E-selectin, ICAM–1 and VCAM–1/INCAM–110 on human endothelial monolayers induced by IL–1. Similar patterns of expression are observed after stimulation of endothelial cells with endotoxin or TNF.

19, 43). Upon activation by thrombin or other mediators, P-selectin is rapidly redistributed to the platelet surface. P-selectin is also found in granules of endothelial cells, known as Weibel-Palade bodies (20, 21). It appears to be targeted to storage/secretory granules by virtue of a sorting signal present in its cytoplasmic domain (44, 45). Endothelial P-selectin, like its platelet counterpart, can be rapidly mobilized to the surface following stimulation. A variety of mediators, including thrombin, histamine, terminal complement components, and H_2O_2 have been shown to stimulate surface expression of endothelial P-selectin (20, 21, 46–48). The expression of P-selectin at the cell surface is short lived, declining substantially within minutes. Recent studies in rodent and canine models suggest that new P-selectin synthesis can be induced by endotoxin and by TNF in a manner similar to that of E-selectin (49–51). Viral infection of endothelial monolayers may result in the expression of P-selectin, apparently through the expression of procoagulant activity and consequent thrombin generation (52).

The function of P-selectin as an adhesion molecule was appreciated after its primary structure had been defined (53, 54). P-selectin expressed on activated platelets has been shown to bind to different types of leukocytes, including neutrophils and monocytes (53, 55, 56). Similarly, endothelial P-selectin can support leukocyte adhesion (52, 54). In addition, purified

P-selectin coated on a surface in vitro supports neutrophil adhesion (57), as well as rolling under the influence of shear stress (58). It is noteworthy that many of the same mediators that stimulate P-selectin expression (e.g. thrombin, histamine, H_2O_2) can cause endothelial retraction and/or damage resulting in increased permeability. In addition, these same mediators stimulate endothelial production of PAF, which triggers leukocyte responses (2–4, 59).

L-Selectin

L-selectin, found on most circulating human lymphocytes, neutrophils, and monocytes, was the first of the selectins to be identified. Initial studies focused on its role in lymphocyte homing to secondary lymphoid tissues (60, 61). Independently, L-selectin was identified as a leukocyte cell surface marker (Leu 8, TQ 1) (62, 63). The connection between the MEL–14 antigen and Leu–8 antigen was made relatively recently (64, 65).

Early studies also implicated the MEL–14 molecule in neutrophil extravasation at sites of acute inflammation (66). More recently, it was demonstrated that L-selectin on neutrophils, monocytes, and lymphocytes can participate in their adhesion to activated endothelium (67–69). This function is best observed when the binding interaction occurs under the influence of fluid shear stress. Studies in vivo have suggested that L-selectin is involved in leukocyte rolling on the vessel wall, a process that can precede firm attachment and extravasation during inflammation (70, 71).

Unlike the other two selectins, L-selectin is constitutively expressed at the cell surface. In an interesting control mechanism, L-selectin is shed from the surface of lymphocytes and neutrophils following cellular activation, a process that may allow leukocytes to detach after the initial adhesive event. (72–75). This process could also affect lymphocyte migration, since L-selectin appears to play a key role in homing to lymph nodes (9, 60, 76). Conformational changes may also help regulate L-selectin function: it has been suggested that activation of neutrophils and lymphocytes by lineage-specific stimuli results in a rapid and transient increase in L-selectin activity prior to its shedding (77).

Selectin Structure

The selectins are identified by their unique domain composition (Figure 2). Amino acid sequences of the three known members are more than 60% identical in the lectin domains (N-terminal 120 amino acid residues) and EGF repeats (13–16, 64, 78). Several studies on structure-function relationships have pointed to the crucial role of the lectin and EGF domains in selectin mediated adhesion. Most adhesion blocking monoclonal anti-

bodies recognize epitopes located within these regions (79–83). Studies with recombinant fusion proteins lacking one or more domains, and with chimeras having mixed selectin domains, support the importance of the two amino-terminal domains in mediating cell adhesion (81, 82). Furthermore, short peptides based on amino acid sequences from separate regions of the lectin domain block leukocyte adhesion to P-selectin (84). It is notable that certain antibodies that bind to the EGF repeat of L-selectin block cellular adhesion without hindering the binding of phosphorylated carbohydrates (see below) (80, 81, 85). Recently, two groups have used site-directed mutagenesis in attempts to identify the amino acid residues of E- (86) and P-selectin (D. Hollenbaugh and A. Aruffo, personal communication) that participate in ligand binding. Both studies suggest a contribution of certain conserved lysine and tyrosine residues found in the lectin domain.

The complement regulatory-like modules found in selectins are approximately 60 amino acids in length and contain six cysteinyl residues, suggesting the formation of three disulfide bonds. Related modules found in complement binding proteins such as complement receptors 1 and 2 contain four cysteines (87–89). The role of the complement regulatory-like modules in selectins is not yet well defined. They likely function, at least in part, to hold the lectin and EGF repeats away from the cell surface, thereby enhancing adhesive function. The importance of this region is suggested by studies with a monoclonal antibody that binds to these modules in L-selectin, recognizes E-selectin, and inhibits the adhesive function of both (90). In addition, L-selectin-immunoglobulin fusion proteins that lack the complement regulatory-like modules exhibit diminished activity (91). To date, no published studies have demonstrated the binding of complement proteins to selectins. On a separate topic, selectins require calcium for activity, as would be predicted by their homology to C-type lectins. In the case of P-selectin, calcium-dependent conformational changes have been demonstrated (92).

The gene for each selectin is organized in a characteristic fashion, with separate exons encoding each of the extracellular domains (38, 93, 94). All three genes have been localized to a cluster on the long arm of human and murine chromosome 1, closely linked to genes for complement binding proteins (38, 78, 80, 95). The cloning of human L-selectin followed immediately upon the cloning of the murine molecule and revealed a highly similar primary structure (64, 78, 96, 97). Recent analysis of cDNAs encoding murine E- and P-selectins, rabbit E-selectin, and canine P-selectin further revealed inter-species conservation of structure and function (41, 49, 51, 98). The number of complement regulatory-like modules in individual selectins may vary slightly between species (49, 98).

Carbohydrate Ligands

Before the primary structures of the selectins were known, some studies had already suggested an interaction of one of the family members with carbohydrates. As described above, lymphocyte adhesion to high endothelial venules of lymph nodes was known to involve the murine lymph node homing receptor (L-selectin) (60). Separate studies had indicated that these adhesive events could be blocked in a specific manner by relatively high concentrations (5–10 mM) of certain phosphorylated monosaccharides such as mannose–6-phosphate (99). In addition, a phosphomannose-containing core polysaccharide of yeast, PPME, was found to be an effective blocker (99). Subsequently, the MEL–14 anti-L-selectin antibody was shown to interrupt the binding of PPME-coated beads to lymphocytes (100). Since the cloning of L-selectin, recombinant proteins have been used to confirm the interaction with PPME (81, 85, 91, 101). In contrast, P- and E- selectin do not appear to bind PPME (81, 102). Early studies also suggested that L-selectin can bind to certain sulfated polysaccharides such as fucoidan (100). Recent work supports this conclusion and suggests that P-selectin binds to fucoidan and heparin (103–106). P- and L-selectin are also similar in that they bind to 3-O-sulfate-galactosyl ceramide (sulfatides) coated on surfaces or incorporated in micelles (105, 106). The precise site of interaction of P- and L-selectin with these structures is not yet defined, and indeed, many proteins that lack structurally defined lectin domains (e.g. growth factors, fibronectin) can bind to these same polyanionic macromolecules.

Many recent studies on selectin-carbohydrate interactions have focused on oligosaccharide structures related to sialylated Lewis x (sLe^x; Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc) and sialylated Lewis a (sLe^a; Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAc) (reviewed in 12). Several articles published by early 1991 identified sLe^x and/or closely related structures as ligands of E-selectin (83, 107–110). It was also demonstrated that P-selectin-dependent rosetting of activated platelets on leukocytes could be blocked by LNF-III, a pentasaccharide containing the Lewis x determinant (Le^x; Galβ1–4(Fucα1–3)GlcNAc) (53). Subsequent studies demonstrated that sialic acid is a component of some P-selectin ligands (102, 111), and that oligosaccharides containing sLe^x are recognized by P-selectin (112, 113). Most recently, evidence was reported suggesting an interaction of murine L-selectin with sLe^x-containing structures (101, 114). Human E- and P-selectin and murine L-selectin have also been shown to interact with molecules containing the sLe^a tetrasaccharide (114–117). It is important to note that the relative binding affinities of the three selectins for sLe^x or related oligosaccharides may differ substantially (12, 103, 118). In addition,

specific modifications of sLe^x and sLe^a resulted in compounds that bound to E-selectin (but not P-selectin) with higher affinity (103, 118).

sLe^x and other fucosylated lactosamines are found in abundance on circulating neutrophils and monocytes (119–124), consistent with the adhesion of these cells to E-selectin. A small percentage of blood lymphocytes are recognized by an anti-sLe^x antibody (115, 124, 125). One monoclonal antibody (HECA–452) that binds to neutrophils, monocytes, and a subset of lymphocytes detects both sLe^x and sLe^a, and perhaps other related E-selectin carbohydrate ligands (30, 115). This antibody identifies a subset of memory T cells that appear to be preferentially recruited to inflammatory-immune reactions in the skin (126, 127). sLe^a is not typically expressed by blood leukocytes but is expressed by certain cancer cells, suggesting a possible role in metastasis (see below).

Several studies have addressed the potential participation of proteins in the cellular ligands of selectins (80, 81, 102, 128–130). For example, an L-selectin-immunoglobulin chimeric protein has been used to affinity purify two sulfated, fucosylated, and sialylated glycoproteins of 50 and 90 kDa from murine lymph nodes (128). A cDNA encoding the 50 kDa glycoprotein predicts a mucin-like molecule containing two serine/threonine rich domains, consistent with substantial O-linked glycosylation (129). The carbohydrate structures on this and other glycoproteins and on glycolipids of lymph node HEV have not yet been well characterized. In separate studies, the peripheral node addressin, thought to be a ligand for L-selectin, has been defined by a monoclonal antibody (MECA 79) that binds to HEV of lymph nodes and inhibits lymphocyte homing (131). This antibody immunoprecipitates multiple glycoprotein species of 50 to 200 kDa, two of which appear to correspond to the 50 and 90 kDa sulfated glycoproteins recognized by murine L-selectin-Ig chimeras described above (85, 128, 132). The MECA 79 antigen can also be detected on endothelium at sites of chronic inflammatory processes (P.R. Streeter and L.J. Picker, personal communication). It seems likely that this antibody recognizes a carbohydrate epitope, although the structure has not yet been characterized.

As noted above, L-selectin appears to participate in the adhesion of neutrophils, monocytes, and lymphocytes to cytokine-activated endothelial monolayers (67–69), possibly through an inducible carbohydrate ligand (68). The nature of the carbohydrate and/or protein components of the L-selectin ligand on cytokine-activated endothelial cells remains to be determined. It is noteworthy that the MECA 79 antigen was not detected in this in vitro setting (P. R. Streeter, L. J. Picker, personal communication). A separate line of investigation has suggested that L-selectin may present carbohydrate ligands to E-selectin and P-selectin (133).

Recent studies also point to a contribution of specific proteins to the

leukocyte ligands of P-selectin. Trypsin treatment of neutrophils and HL60 cells abrogates their adhesion to P-selectin (but not E-selectin) as well as their capacity to bind solution-phase P-selectin (102, 134, 135). Further, P-selectin has been used to affinity purify a glycoprotein (Mol wt ~120,000 reduced) from leukocyte extracts (130). Thus, both L- and P-selectin appear to bind to a relatively small number of cell surface glycoproteins. The nature of the participation of protein and carbohydrate in these selectin ligands has not yet been established. In addition, the relative contributions of high versus low affinity binding sites to cell adhesion in vitro and in vivo are not yet known.

In summary, E-, P-, and L-selectin bind to one or more types of carbohydrates, including sLe^x-related structures, sulfated polysaccharides (heparin, fucoidan), and phosphated mono- and polysaccharides (mannose–6-phosphate, PPME). In addition, specific cell proteins may participate in the presentation of selectin ligands.The molecular details of selectin-ligand interactions (e.g. binding sites and bond formation) have not yet been determined. A corollary to this statement is that the binding of individual selectins to different carbohydrate types may or may not be by the same mechanism. A complete description of these interactions will require determination of binding affinities and rate constants, as well as structural analysis by x-ray crystallography.

IgCAM-INTEGRIN INTERACTIONS

Bimolecular interactions between immunoglobulin-related cell adhesion molecules (IgCAMs) and integrins occur with great frequency and significance throughout the immune system (reviewed in 5, 10, 136, 137). Moreover, the specific contribution of receptor/counter-receptor pairs within these families to endothelial-leukocyte interactions has been the topic of intensive research (5, 6, 9, 76, 137). It is now widely accepted that endothelial cell surface IgCAMs, including ICAM–1, ICAM–2, and VCAM–1/INCAM–110, play key roles in adhesion and transmigration of blood leukocytes. They accomplish these tasks through direct binding to leukocyte cell-surface integrins.

ICAM–1 and –2 and their Counter-Receptors CD11/CD18

Two of the members of the Ig superfamily expressed by vascular endothelial cells, designated intercellular adhesion molecule–1 (ICAM–1; CD54) and intercellular adhesion molecule–2 (ICAM–2), are closely related in structure and function (reviewed in 5). These transmembrane glycoproteins contain five and two extracellular Ig domains, respectively (138–140). The two Ig domains of ICAM–2 are most homologous to the

two amino-terminal domains of ICAM–1, which are known to participate in cell-cell interactions (140, 141). Both ICAM–1 and ICAM–2 are bound by one or more of the leukocyte cell surface $\beta 2$ integrins (5, 138, 140, 142–144), a group of three heterodimeric molecules that share a common β subunit (5, 10, 137).

Like E-selectin, ICAM–1 is expressed in abundance on vascular endothelium after several hours of stimulation by IL–1 or TNF (145, 146) (Figure 3). However, the pattern of expression of ICAM–1 differs from that of E-selectin in several ways (reviewed in 2). First, a low level of ICAM–1 is typically found on unstimulated endothelial cells. Second, IL–1- or TNF-induced expression of ICAM–1 occurs more slowly and is prolonged by comparison to that of E-selectin. Third, interferon-γ induces ICAM–1 expression but not E-selectin expression. ICAM–1 is also expressed in an inducible manner on a wide variety of other cell types, and may thereby contribute to adhesive events in many settings (5). ICAM–2 expression on vascular endothelial cells does not appear to be regulated by cytokines (140, 147). The constitutive expression of this molecule on endothelium provides a ready surface for $\beta 2$ integrin-dependent adhesion of leukocytes. This may be particularly important in certain rapidly occurring inflammatory events.

Endothelial cell surface ICAM–1 and ICAM–2 appear to contribute to the adhesion and transmigration of most leukocyte types through an interaction with $\beta 2$ integrins. Neutrophils, monocytes, lymphocytes, and natural killer (NK) cells express CD11a/CD18 (LFA–1), which has been shown to bind both ICAM–1 and ICAM–2 (5, 6,137). In addition, neutrophils, monocytes, and NK cells (but not most lymphocytes) express CD11b/CD18 and CD11c/CD18, which are found at the cell surface as well as in storage granules (5, 6,137). The expression and function of $\beta 2$ integrins are tightly controlled. Activation of leukocytes by a variety of mediators results in a transient increase in adhesion by CD11/CD18-dependent mechanisms. This increased adhesive capacity occurs through qualitative changes in CD11a/CD18 function and through quantitative changes in the expression of CD11b/CD18 and CD11c/CD18 (5, 6,137).

During endothelial-leukocyte adhesion events, a variety of factors may stimulate CD11/CD18 activity. For example, endothelial cell surface associated PAF, a potent lipid-based leukocyte stimulator, is upregulated within minutes following treatment by thrombin and other mediators (2, 3). In addition, endothelial cells synthesize and secrete the leukocyte activator IL–8 and related low molecular weight inflammatory cytokines in response to endotoxin, IL–1, and TNF. It has also become clear that engagement of a variety of leukocyte cell surface molecules capable of signal transduction can alter $\beta 2$ integrin function. For example, T cell

receptor binding to antigen-MHC complexes results in a substantial increase in the activity of CD11a/CD18 (148). In addition, it has been suggested that the molecular interaction of E-selectin with leukocyte cell surface ligand(s) stimulates CD11/CD18 function (149, 150). In this regard it is interesting to note that the carbohydrate sLe^x, one known ligand of E-selectin, is expressed on a variety of neutrophil cell surface glycoproteins, including CD11/CD18 molecules themselves (151), L-selectin (133), and CD66, a member of the Ig superfamily (152). Other bimolecular interactions that involve leukocyte cell surface CD2, CD31, and CD44 could also up-regulate the ability of CD11/CD18 molecules to bind endothelial cell surface ICAM–1 and ICAM–2 (see below).

Rapid and transient modulation of CD11/CD18 activity provides a mechanism for local control of adhesive events at the vessel wall on a short-term basis. It should also be noted that memory T cells express more of several adhesion molecules, including CD11/CD18, than do naive T cells, and are preferentially recruited to inflammatory sites by comparison to naive T cells (5, 9,60). Thus, longer term effects on the expression and function of CD11/CD18 molecules may also be important in regulating adhesion. In conjunction with short-term and long-term regulation of endothelial cell expression of adhesion molecules, these leukocyte-directed regulatory mechanisms allow for the fine tuning of inflammatory and immunological responses.

VCAM–1/INCAM–110 and the α4β1 Integrin

By 1989 the roles of E-selectin and ICAM–1 in the adhesion of leukocytes to endothelial cells were becoming well established. At that time, at least two lines of evidence pointed to the existence of a third cytokine-inducible endothelial adhesion molecule. First, it was apparent that the adhesion of most peripheral blood lymphocytes to activated endothelium could not be fully explained by E-selectin and ICAM–1 (153). Second, certain non-lymphoid tumor cells (e.g., melanomas) were found to adhere to activated endothelium by mechanisms that appeared to be independent of E-selectin and ICAM–1 (154). In this setting, a monoclonal antibody generated against TNF activated endothelium was found to block the adhesion of both melanoma cells (155) and blood lymphocytes (156). This antibody identified a cytokine-inducible endothelial glycoprotein of 110 kDa that was immunochemically distinct from previously described structures (155–157); it was designated inducible cell adhesion molecule–110 (INCAM–110). By immunohistochemistry, INCAM–110 was found to be expressed in a variety of tissues, both vascular and non-vascular (157). Other investigators isolated a cDNA clone from cytokine activated endothelial cells

by differential subtraction that encoded a six-Ig domain molecule that supported the adhesion of lymphocytes (158). The molecule encoded by this cDNA was named vascular cell adhesion molecule–1 (VCAM–1). Shortly thereafter, these two experimental approaches intersected when it was found that the anti-INCAM–110 antibody reacted with cells transfected with the cDNA encoding VCAM–1 (132, 156). The name VCAM–1 is more commonly used.

Endothelial cells respond to IL–1 and TNF by upregulating the expression of VCAM–1, with maximal activity reached by 6–12 hours (155, 156, 159–161). Like ICAM–1, the high level of expression is prolonged by comparison to that of E-selectin (Figure 3). Interestingly, the cytokine IL–4 acts on endothelial cells to increase the expression of VCAM–1 but not E-selectin or ICAM–1 (162, 163). VCAM–1 is also expressed on several non-vascular cell types, including populations of dendritic cells found in lymph node and skin, bone marrow stromal cells, and synovial cells in inflamed joints (157, 164–167). Analysis of 5′ flanking sequences in the human VCAM–1 gene has revealed the presence of two NFκB sites as well as other functional elements (168, 169).

Endothelial VCAM–1 was first shown to support the adhesion of lymphocytes and monocytes (but not neutrophils) through an interaction with the integrin $\alpha 4\beta 1$ (VLA–4; CD49d/CD29) (29, 156, 159, 160, 170), which also binds to fibronectin (171, 172). In addition, eosinophil and basophil adhesion to activated endothelium appear to involve VCAM–1 (35, 163, 173, 174). Outside the vasculature, VCAM–1 participates in a variety of lymphocyte adhesive events, including the binding of B cells to lymph node follicular dendritic cells (175), and the adhesion of lymphocyte precursors to bone marrow stromal cells (164, 165). Recent evidence suggests that these adhesive interactions influence other lymphocyte functions. For example, molecular engagement of $\alpha 4\beta 1$ on T lymphocytes by VCAM–1 has been shown to act as a costimulant of proliferation (176, 177). In a separate arena, a recent study indicates that VCAM–1 and $\alpha 4\beta 1$ are expressed on immature muscle cells and play a role in myogenesis (178).

After the initial cloning of VCAM–1, it was recognized that the predominant endothelial form of this molecule contains seven Ig domains, including an alternatively spliced fourth domain (179–181). Among the seven Ig domains, the N-terminal domain (domain 1) and the fourth domain are highly similar in amino acid sequence, and appear to function in leukocyte adhesion (182, 183). cDNA clones from other species, including mouse, rat, and rabbit, demonstrate substantial conservation of amino acid sequence (similarity $\geqslant 70\%$) and functional interactions with $\alpha 4\beta 1$ (184, 185).

Mucosal Addressin and the α4β7 Integrin

Lymphocyte homing to secondary lymphoid tissues has been the subject of recent reviews (60, 186). The terms "homing receptor" and "addressin" are used to refer to cell surface adhesion molecules on lymphocytes and endothelium (typically in HEV) respectively, that contribute to tissue-specific localization of lymphocytes (60, 186). As previously noted, lymphocyte L-selectin appears to function as a homing receptor for peripheral lymph nodes. In this setting, L-selectin is thought to bind to the peripheral node addressin, a structure identified by antibody MECA 79 which may be a carbohydrate epitope (131). Other molecules appear to direct lymphocyte traffic to gut-associated mucosal lymphoid tissue (e.g., Peyer's patches). In recent studies it has been demonstrated that a lymphocyte cell surface integrin designated α4β7 can support adhesion to mucosal HEV (187, 188). This integrin has the same α subunit as the counter-receptor for VCAM–1, α4β1, described above. The α4β1 integrin may also participate in lymphocyte homing to mucosal tissue (188). Other reports suggest that α4β7 binds to VCAM–1, but this is controversial (189, 190).

In separate studies, a monoclonal antibody (MECA 367) generated against gut-associated lymphoid tissue was found to bind mucosal HEV and to block the adhesion of a population of lymphocytes. Using this antibody, a 58–66 kDa glycoprotein, designated the mucosal addressin, was purified from mucosal tissue (191–194). When immobilized onto a surface, the mucosal addressin supports lymphocyte adhesion (192). A recently isolated cDNA clone encoding the mucosal addressin has revealed that it is a member immunoglobulin superfamily, and has been used to demonstrate a direct molecular interaction with the α4β7 integrin (M. Briskin and E. Butcher, personal communication). Under certain circumstances, the mucosal addressin may also be found in non-mucosal tissues, suggesting additional functional roles. For example, recent studies suggest that it is expressed by endothelial cells at sites of recurrent episodes of experimental allergic encephalomyelitis (195, 196).

OTHER MOLECULAR INTERACTIONS

In addition to the receptor/counter-receptor pairs described above, a variety of other cell-surface molecules participate in endothelial-leukocyte interactions. In some cases, their direct contribution to adhesion is uncertain, although their structure and other functions may be very well defined (e.g., MHC molecules).

CD31 (PECAM–1, endoCAM)

CD31 is a transmembrane glycoprotein of ~130,000 daltons found on endothelial cells, platelets, neutrophils, monocytes, and subsets of T cells (197). A member of the immunoglobulin superfamily of genes, its cDNA sequence predicts a transmembrane protein containing six Ig domains (198) (Figure 4). Transfection studies suggest that this molecule can mediate cell-cell adhesion through both homophilic (CD31-CD31) (199), and heterophilic (CD31-unknown counter receptor) binding interactions (200). CD31 is constitutively expressed in abundance on endothelium in vitro and on all continuous endothelial linings in vivo (201). When endothelial cells come into contact to form a cobblestone-like monolayer, CD31 redistributes to the cell borders and is thought to participate in the endothelial cell-endothelial cell interactions that limit vascular permeability. As noted in the introduction, increased permeability is a characteristic component of inflammation. It has been recognized that chemoattractive agents alone will promote little leukocyte influx in the absence of factors that increase vascular permeability (reviewed in 2). In this regard, mediators such as thrombin and histamine that act on endothelium to cause cellular retraction, may do so at least in part through an effect on CD31. These mediators stimulate phosphorylation of serine residues in the cytoplasmic region of CD31 which may modulate cytoskeletal interactions (202, 202a).

The role of CD31 in the adhesion and transmigration of neutrophils, monocytes, and T cells on endothelium deserves further attention. Homophilic binding interactions between leukocyte CD31 and endothelial CD31 could be particularly important during transmigration, when the leukocyte should encounter the highest density of endothelial CD31 molecules (201). Recent experiments have suggested that engagement of leukocyte CD31 results in signal transduction that increases adhesion mediated by $\beta 1$ and $\beta 2$ integrins (203–205). Thus, the interaction of leukocyte CD31 with endothelial CD31 or with another counter-receptor could promote adhesion through leukocyte CD11/CD18- or $\alpha 4\beta 1$-dependent mechanisms.

LFA–3–CD2 Interactions

Interactions of lymphocyte cell surface CD2 and target cell LFA–3 (CD58), both members of the immunoglobulin superfamily (136), are thought to be important in a variety of immunological functions (reviewed in 5, 136). The effectiveness of this receptor-counter receptor pair in mediating cellular adhesion is highly influenced by cell surface charge (5). For example, activated lymphocytes that express less sialic acid than their

resting counterparts demonstrate greater adhesion via LFA–3/CD2-mechanisms (5). Vascular endothelial cells express LFA–3 (Figure 4), but there has been little direct evidence of a substantial role for this molecule in adhesion (5, 76). However, it is well appreciated that endothelium can present antigen to lymphocytes and can support lymphocyte activation, processes which are blocked significantly by anti-LFA–3 antibody (reviewed in 76).

MHC Molecules CD4, CD8 Interactions

Perhaps no bimolecular interactions have received as much attention as those that occur between lymphocyte cell surface CD4 or CD8 molecules and MHC molecules on antigen presenting cells and target cells (5, 136, 206, 207). Despite substantial scrutiny, the role of these molecules in adhesion remains controversial (5, 76). Activation of endothelial cells by interferon-γ results in increased class I MHC expression and in de novo expression of class II MHC molecules; TNF may augment these responses (76). These cytokine treatments also result in increased lymphocyte adhesion, although the contribution of endothelial MHC molecules to adhesion remains poorly defined (5, 76). On the other hand, the role of these molecules in endothelial cell presentation of antigen to lymphocyte

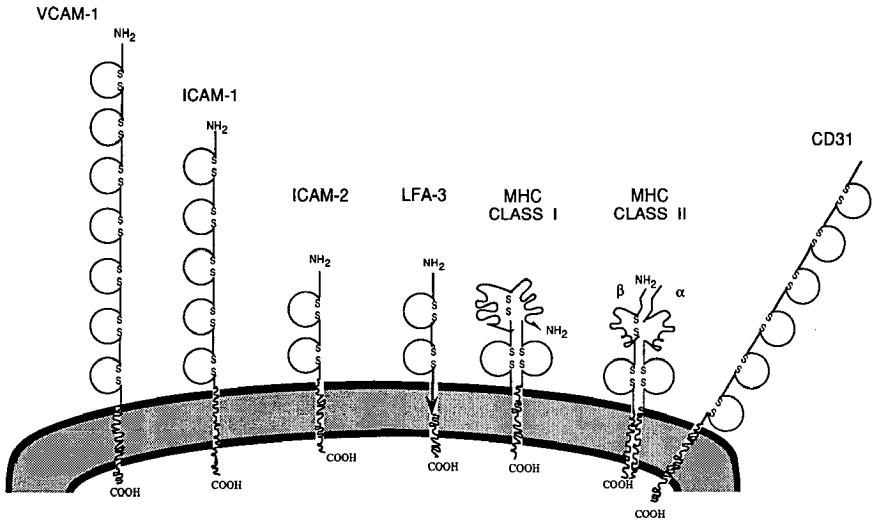

Figure 4 Endothelial cell members of the immunoglobulin gene superfamily thought to interact with leukocytes. LFA3 (CD58) can be expressed in transmembrane and phosphotidylinositol-linked forms. CD31 is found in abundance at the borders of adjacent endothelial cells, where it is thought to participate in endothelial cell–endothelial cell interactions.

is well established (76). Interestingly, engagement of the T cell receptor, a process that involves specific antigen in association with MHC molecules, results in increased function of CD11a/CD18, the leukocyte integrin that binds to ICAM–1 and ICAM–2 (148).

VAP–1

A recent study using a monoclonal antibody-based approach has defined what appears to be a new endothelial cell surface adhesion molecule, designated vascular adhesion protein 1 (VAP–1) (208). Immunohistochemical studies indicated that VAP–1 is expressed by HEV in several locations, including tonsil, peripheral lymph node, and inflamed synovium. An anti-VAP–1 monoclonal antibody blocked adhesion of lymphocytes to these HEV in a modified Stamper-Woodruff assay and was used to immunoprecipitate a 90-kDa glycoprotein capable of supporting lymphocyte adhesion. N-terminal sequence analysis revealed no significant homology to known molecules; the complete primary structure of VAP–1 is not yet known. The leukocyte cell surface counter receptor for VAP–1 and its relationship to the other molecules described in this review remain to be determined.

Leukocyte Molecules with Putative Endothelial Ligands

CD44 (pgp1, Hermes, H-CAM, ECMRIII) has an interesting and complex history (reviewed in 209–211). Molecular cloning of this molecule suggested a relationship to cartilage link proteins (212–215), and subsequent studies revealed multiple isoforms that arise by alternative splicing, and which can have different amounts of attached glycosaminoglycans (209). CD44 has been shown to interact with several cell surface and matrix components, including fibronectin, collagen, and hyaluronate, a non-sulfated glycosaminoglycan (216–218). CD44 is found on epithelial cells, leukocytes, keratinocytes, and a variety of tumors; cellular distribution of the individual isoforms varies. A soluble form has been found in blood and synovial fluid (209). Antibodies to CD44 have been reported to block lymphocyte adhesion to mucosal HEV and to activated endothelium (219–221). A specific endothelial ligand for CD44 has not yet been identified.

CD14, a member of a family of leucine-rich proteins (222, 222a, 222b), is found in abundance on monocytes and in smaller amounts on neutrophils and appears to participate in a variety of cellular functions, including the response to endotoxin (223). A recent study found that a monoclonal antibody directed against CD14 could partially inhibit monocyte adhesion to IL–1-activated endothelial monolayers (224). The precise molecular interactions involved in this effect are not yet known. Other studies indicate

that engagement of CD14 may alter the function of monocyte CD11/CD18 molecules (225, 225a).

The molecules described above seem sufficient to enable endothelial-leukocyte adhesion in a wide variety of settings. Future studies should provide important new information regarding the regulation of their expression and function. Of course, other receptor/counter-receptor pairs may yet be identified.

ENDOTHELIAL-LEUKOCYTE ADHESION MOLECULES IN VIVO

Endothelial-leukocyte adhesion molecules work in concert with cell-associated and soluble mediators to direct the movement of blood leukocytes. Faced with invading microorganisms, toxic substances, or tissue injury, the body mobilizes a potent defense (1). In most cases, neutrophils arrive first and begin neutralizing the offending stimulus (e.g. phagocytosis of bacteria). Soon thereafter, lymphocytes join the effort with their weapons of specific immunity. This response is usually successful. In the immunocompromised host, however, defense systems may be overwhelmed. Finally, there are times when the defensive response itself becomes part of the problem. Leukocyte-mediated tissue damage is a major component of a variety of human disease processes, including adult respiratory distress syndrome (ARDS), autoimmune diseases, graft rejection, and the post-ischemia reperfusion injury that occurs after thrombolytic therapy opens an occluded coronary artery.

Expression

Information regarding the expression of endothelial-leukocyte adhesion molecules in vivo is accumulating rapidly. Some studies have used simple models of inflammation in animals and human volunteers. Others have documented molecular expression at one or more stages of specific human diseases. Both approaches have yielded valuable insights. The earliest study on E-selectin in situ revealed a time-dependent induction of expression at a site of a delayed hypersensitivity reaction (DHR) in human skin (27). In a simple animal model of inflammation, injection of endotoxin into the skin of a baboon resulted in early (peak at 2–4 hr) and transient expression of E-selectin that correlated with the influx of neutrophils; ICAM–1 expression did not change appreciably (226). In the same model, TNF caused a more robust induction of E-selectin, as well as an increase in ICAM–1 expression that was delayed (onset at 9 hr) and prolonged by comparison to that of E-selectin (227). The expression of E-selectin and ICAM–1 were paralleled by an accumulation of neutrophils and mono-

nuclear leukocytes, respectively. Combinations of cytokines caused more complex responses (227). In several other models of skin inflammation, E-selectin expression was found to correlate with neutrophil influx (228, 229). However, E-selectin can be detected in settings where few neutrophils are found (27, 230, 231). In certain studies, VCAM–1 expression correlates with increased mononuclear leukocyte infiltrates (228, 230, 232), consistent with its ability to support the adhesion of lymphocytes and monocytes but not neutrophils. Although most studies have focused on microvascular endothelium, VCAM–1 has also been found on rabbit aortic vascular endothelium early in the development of atherosclerotic lesions (233). Other studies have identified ICAM–1 on several populations of cells within the atherosclerotic lesions themselves (234, 235).

Numerous recent studies have demonstrated the expression of E-selectin, VCAM–1, and ICAM–1 during inflammatory disease processes (for examples see 157, 230, 233, 235–242). Several general patterns have emerged. In resting tissues, most endothelial cells express little or no E-selectin and VCAM–1. In contrast, ICAM–1 is typically expressed at a modest level in most vessels and is found on a variety of nonvascular cell types. At active sites of inflammation, expression of endothelial cell E-selectin and VCAM–1 is increased according to the nature and intensity of the stimulus. Endothelial ICAM–1 expression may also be enhanced above resting levels. VCAM–1 and ICAM–1 expression on nonvascular cell types (e.g. dendritic cells) is also increased. Induced endothelial E-selectin and VCAM–1 are most readily detected in postcapillary venules (243), the site of prominent vascular leak and leukocyte extravasation during inflammation. In certain animal disease models, such as septic shock, E-selectin has also been found on capillary endothelium (244); VCAM–1 is seen relatively frequently on the endothelium of capillaries and arterioles at sites of inflammation (157, 228).

In the study of endothelial-leukocyte adhesion molecules, it is tempting to define functional groups or subsets of endothelial cells based on adhesion molecule expression, similar to those described for lymphocytes. This would imply specific, stable combinations of adhesion molecules. However, most data suggest that endothelial expression of inducible adhesion molecules is a dynamic process that occurs in response to specific mediators. Molecules are expressed over a wide range of cell surface densities, depending on individual mediators and duration of exposure. In cooperation with constitutively expressed endothelial molecules (e.g. ICAM–2, LFA–3, CD31) and endothelial-derived mediators (e.g. PAF, IL–8), the inducible adhesion molecules effect leukocyte adhesion and extravasation. The pattern of expression of adhesion molecules appears to influence the cellular makeup of the inflammatory infiltrate. For example,

it has long been appreciated that neutrophils predominate in most early inflammatory lesions, whereas monocytes and lymphocytes constitute the majority of extravasating cells at later time points. This may be explained in part by the temporally distinct expression of E-selectin and VCAM–1 as well as by the local mix of cellular mediators. In particular settings, combinations of adhesion molecules and mediators may lead to markedly enhanced recruitment of one leukocyte type (e.g. eosinophils in allergic inflammation).

Function

Recent studies using animal models have dramatically illustrated the participation of endothelial-leukocyte adhesion molecules in acute inflammatory processes. For example, an antibody directed against E-selectin was found to block neutrophil extravasation and vascular leak in the lungs of rats following IgG immune complex induced damage (245). Interestingly, IgA immune complex induced damage, which appears to involve mononuclear leukocyte infiltrates (rather than neutrophil infiltrates), was not blocked by anti-E-selectin antibodies, while an antibody against the leukocyte β subunit CD18 was highly protective (246). Other studies have also demonstrated a role for CD18 and ICAM–1 in models that involve neutrophil-dependent mechanisms in pulmonary damage (247–250). Anti-P-selectin antibodies have been found to protect against neutrophil-dependent acute lung injury that occurs after intravenous activation of complement by cobra venom factor (251). In a primate model of extrinsic asthma, antibody blocking data have suggested that E-selectin plays a major role in neutrophil influx associated with late phase airway obstruction (252). Both E- and P-selectin may participate in the neutrophil-mediated damage associated with ischemic reperfusion injury in the heart (51, 253). Antibodies to L-selectin and soluble forms of L-selectin have been used to demonstrate the participation of this molecule in a variety of animal models of homing and inflammation (61, 66, 254).

Lymphocyte-mediated tissue damage occurs in transplant rejection and in autoimmune diseases. A recent study suggests that expression of VCAM–1 on blood vessels within human cardiac allografts is indicative of rejection (232). In separate studies, cardiac allografts in mice survived indefinitely (>80 days) when the animals were treated for six days following transplantation with antibodies against ICAM–1 and CD11a/CD18 (255). This remarkable effect appeared to depend on the development of specific tolerance for the foreign tissue. Notably, antibodies against either ICAM–1 or CD11a/CD18 alone had little effect. Other studies in a murine model have indicated that antibodies against this receptor/counter-receptor pair may prove to be a useful therapy in the treatment of graft

versus host disease (256). Finally, antibodies against the $\alpha 4\beta 1$ integrin were found to prevent experimental autoimmune encephalomyelitis in rats, an inflammatory condition of the central nervous system with many similarities to human multiple sclerosis (257).

It is interesting to note that soluble forms of several endothelial-leukocyte adhesion molecules have been found in the circulation. As described above, activated leukocytes shed L-selectin. In addition to shed L-selectin, soluble forms of P-selectin, perhaps synthesized from alternatively spliced mRNA, have been proposed (14, 57, 93). Soluble forms of L-selectin, P-selectin, and ICAM–1 in circulation have been reported (258–260). The possibility that these circulating forms of adhesion molecules alter inflammatory responses deserves further attention.

A POSSIBLE ROLE IN CANCER METASTASIS

Recent studies have suggested that certain nonlymphoid tumor cells (e.g. melanoma and carcinoma) may interact with endothelial-leukocyte adhesion molecules during hematogenous metastasis. Initial studies demonstrated that tumor cells bound in increased numbers to cytokine-activated endothelium, a process reminiscent of endothelial-leukocyte adhesion (154, 261, 262). Melanoma adhesion to activated endothelium appeared to be independent of E-selectin and ICAM–1 (154). INCAM–110 (VCAM–1) was identified as a cytokine-inducible endothelial cell surface glycoprotein that mediates melanoma adhesion (155) through an interaction with the $\alpha 4\beta 1$ integrin (132). Selectin-carbohydrate interactions have also been implicated in the hematogenous spread of cancer cells. Early work demonstrated that endothelial E-selectin could support the adhesion of human colon cancer cells (155). The carbohydrate structures of sLe^x and sLe^a are expressed in abundance on most human colon cancers and appear to participate in this adhesive interaction (263–266). Separate studies have demonstrated that distinct isoforms of CD44 expressed on tumor cells may alter metastatic capacity and growth (267–269). Other endothelial-leukocyte adhesion molecules will likely also be found to participate in the metastatic process.

CONCLUDING REMARKS

At the boundary between blood and tissue lies the vascular endothelium. From this unique position, it plays a key role in the regulation of a variety of pathophysiological processes. Its contributions to the development of inflammatory and immunological reactions are now well established. Among its many functions, none has received more attention than the

controlled expression of specific cell surface molecules that support leukocyte adhesion. The burgeoning knowledge in this area invites the creation of organizational categories. Too often, these categories are based on minor distinctions rather than on major similarities. Although adhesion of leukocytes to the blood vessel wall happens in many settings throughout the vasculature, the underlying mechanisms are the same. In response to specific mediators, vascular endothelial cells can express a handful of cell surface molecules capable of supporting leukocyte adhesion. If the number of molecules is sufficient, the physical conditions (e.g. shear forces) are permissive, and the leukocytes are responsive, adhesion will occur. Maybe.

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