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IMMUNOLOGY AND EXPERIMENTAL DERMATOLOGY

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INTRODUCTION

This account is only incidentally biographical. Its aim is to relate the successive steps that led to an understanding of contact-type reactions and certain other allergic responses of guinea pigs. This narrative reflects the chanciness with which important observations come to light, so often attributed to the "prepared mind"—whatever that phrase really signifies.

The story starts in 1932, when, at age 27, I was hired by Simon Flexner to work with Karl Landsteiner, then ten years at The Rockefeller Institute for Medical Research. Landsteiner's work was three-fold. He continued, first of all, his work on human blood groups. Secondly, he was investigating hapten-protein complexes which induced antibodies in rabbits and appeared to be hapten-specific; this led to his study of inhibition tests, in which *p*-amino-benzoyl-hapten would block precipitation of hapten-carrier$_2$ by antibodies against hapten-carrier$_1$; the respective carriers were essentially non–cross-reacting. Third, he was starting work on synthesis of glycyl- and leucyl- peptides to provide di-, tri-, pentapeptides for antigenic specificity. Still only a gleam in Landsteiner's eye was the possibility of studying contactant reactivity in the laboratory. In 1907, without any hard evidence, Wolff-Eisner (1) had voiced the concept that dermatological problems result from complexing of medicaments with the patient's tissues ("self"). A few years before my arrival, poison ivy leaves had been collected,

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dried, extracted, and used to treat rabbits. The animals remained unscathed, though not the laboratory personnel.

The first important breakthroughs in the study of allergic responses came on the European continent. The Russian Wedroff in 1927 (2, and further papers) reported that compound 2:4 dinitrochlorobenzene (DNCB)[2] would sensitize humans. In Switzerland with Steiner-Wourlisch, Bloch sensitized human subjects with crystalline material isolated from primula leaves (1926) and later they found guinea pigs susceptible (1930). In parallel, Rudolf Mayer used p-phenylenediamine salve to sensitize first human volunteers (1928) and then (4) guinea pigs (1931).

At a Congress in Rome in 1933, Landsteiner talked with the renowned Wilhelm Kolle, who suggested repeated intradermal (i.d.) injections of very tiny amounts of DNCB.[1] That fall, the problem was given to John L. Jacobs. Indeed, he learned that 2.5 μg amounts of DNCB sensitized guinea pigs well, as confirmed by their response to a later i.d. injection. Shown the animals, Peyton Rous was convinced of their significance but complained that the average size of the intradermal test reaction was "too small." Trained also as a chemist, Landsteiner wrote to Holland, where Hollemann and colleagues had synthesized a large series of nitro- and chloro-substituted benzene ring compounds, and Hollemann kindly shipped many preparations. Seven of seventeen such compounds were found to sensitize guinea pigs by i.d. injection, and all of these had one substituent on the ring that could be split off sufficiently to permit covalent linkage with the organic base aniline (5) used as model of ε-NH_2-groups of lysine. Thus Wolff-Eisner's concept, mentioned above (1), proved correct. During later work on sensitization with a very large number of chemicals, the usefulness of *contact* testing, as used in humans, became apparent.

Jacobs left Landsteiner's laboratory for Boston in 1936, and I was asked to assume this area of research. For four years I had been working on other types of problems and learning much in that industrious atmosphere.

At that time, however, nothing was really understood about sensitization. A few sensitized guinea pigs possessed anaphylactic reactivity to hapten-protein conjugates, usually mild, but Landsteiner was delighted with every positive reaction since he conceived of antibody and cell-bound antibody as the result of hapten-self structures. I injected DNCB into the

[2] Abbreviations used: BSA, bovine serum albumin; CFA, complete Freund's adjuvant (containing mycobacteria); DMSO, dimethylsulfoxide; DNCB, 2:4 dinitrochlorobenzene; DNFB, 2:4 dinitrofluorobenzene; DNP-, dinitrophenyl-; GPA, guinea pig albumin; IFA, incomplete Freund's adjuvant (lacking mycobacteria); PA, picric acid; P.O., paraffin oil; PPL, poly-L-lysine; TNCB, 2:4:6 trinitrochlorobenzene (picryl chloride); TNP-, trinitrophenyl-.

tongue of the guinea pig and into many parenteral sites; always the conclusion seemed inescapable that only the i.d. route was effective. The burning question of the time concerned the way in which the *whole skin* acquires sensitivity. To test a current hypothesis that cell-bound antibody crept through the epidermis by its intracellular bridges, Schreus and his colleagues (6, 7) had prepared skin islands on guinea pigs to see if a break in the continuity of epithelium would block the spread. They reported that the spread was indeed blocked. We got into the act with poison ivy extract; at first, Schreus appeared to be right. Then I demonstrated that it was possible to interrupt epidermal continuity yet leave intact the underlying panniculus carnosus muscle, along which one or two patent lymphatic channels joined the isolated epidermal area with deeper lymphatics. In this situation, ivy extract applied to the island would sensitize the whole animal. We erred, however, in our assumption that it was the sensitizer which escaped via lymphatics. Thirty years later, we would reverse our early thinking and say instead that lymphocytes arrive easily at an island via the blood vessels, but patent lymphatics are needed to allow the lymphocytes to home to a lymph node after experiencing the allergen locally.

Jules Freund, following up the studies of Coulaud and of Saenz in France, had just (1937) confirmed Saenz strikingly—injection of *dead* mycobacteria in paraffin oil leads to high sensitivity to tuberculin. Freund provided a sample and I undertook intraperitoneal (i.p.) injection with it, following the next day with i.p. picryl chloride (2:4:6 trinitrochlorobenzene, TNCB); one further similar cycle was given—and contact sensitivity arose (to Freund's surprise). Substitution of simple TNCB by picrylated stromata of guinea pig erythrocytes in the same routine was also effective (8). The spaced injections were abandoned when Freund introduced his water-in-oil emulsion form, and injections of emulsion were made into the nuchal muscles. I grew my own organisms and used fewer bacterial cells than in the conventional Complete Freund's Adjuvant (CFA). Shortly afterward we observed that further contact tests on animals treated with the emulsion would cause a pronounced increase in sensitivity—I called this the "combination method." I had been developing a superb line of albino guinea pigs with a broad gene pool and high susceptibility for sensitization. Years later, the grapevine whispered that my results were not confirmed when Hartley guinea pigs were used. I found indeed a strain difference, for only about 20% of Hartley animals resembled my own stock in response to the Combination Method.

The three fundamental sensitizing procedures—i.d. injection, absorption through the skin, and use of mycobacteria with paraffin oil, and various minor modifications—are summarized in Table 1.

Table 1 Methods of sensitizing guinea pigs to allergenic chemicals

Techniques	Year introduced
Injections i.d., microgram amounts in banal solvents on several occasions	Kolle, 1933[a]; Landsteiner & Jacobs, 1936[b]; Draize *et al.*, 1944[c]; Food and Drug Administration, 1959[d]
Percutaneous Absorption	
Salves (Primula, *p*-phenylenediamine)	Bloch & Steiner-Wourlisch, 1930[e]; Mayer, 1931[f]
Local irritation of application site	
DNCB, 5%, repeatedly, highly irritating	Schreus, 1938 with Schreiber & Müller, 1938[g]
DNCB, 2% in alcohol repeatedly	Landsteiner & Chase, 1938 (Chase, 1947)[h]
Organic peroxides added to allergen	Landsteiner & diSomma, 1938[i]
Site prepared with cantharidin	Landsteiner & diSomma, 1940[j]
Application on contact site of an unrelated sensitivity	Landsteiner & Chase, 1941[k]
Freon 12 on human subjects	Kligman & Epstein, 1959[l]
Closed patches	Buehler, 1965[m]; Ritz & Buehler, 1980[n]
Sodium lauryl sulfate to mascerate skin	Magnusson & Kligman, 1970[o]
Detergents with beryllium fluoride	Polák, Barnes, Turk, 1968[p]
Dry ice burns	Maguire, 1973[q]
DMSO pretreatment before topical application	Maguire, 1974[r]
Cyclophosphamide pretreatment enhances	Maguire & Ettore, 1967[s]
DNCB sensitivity	Hunziker, 1968[t]
Cyclophosphamide removes suppressor cells	Polák & Rinck, 1977[u]
Use of mycobacterial adjuvant	
Mycobacteria in P.O. i.p., allergen i.p., independently	Landsteiner & Chase, 1940[v]
Mycobacteria in P.O. and hapten-homologous RBC stromata i.p., independently	Landsteiner & Chase, 1941[w]
Above procedure, later boosting by contact tests	Chase, 1942 (Chase, 1954)[x]
Aqueous chemical, mycobacteria in P.O., W/O emulsifier combined as CFA	Freund & McDermott, 1942[y]
Allergen in hydrocarbon phase	Landsteiner & Chase, 1940[v]
Allergen in aqueous phase	Benacerraf & Gell, 1959[z]
Hapten-protein conjugates in CFA in footpads	Benacerraf & Levine, 1962[aa]
Combination Method	
Hapten-stromata conjugate in CFA type of emulsion i.m., then boosting by contact tests, days 10, 17	Chase, 1942 (Chase, 1954)[x]

Table 1—*continued*

Techniques	Year introduced
Split-adjuvant Technique	
Mycobacteria in P.O. i.d., at 24 hr. allergen i.d. to sites or topically over sites	Maguire & Chase, 1967[bb]
Hapten-epidermal extracts with IFA into footpads	Salvin & Smith, 1961[cc]
Hapten-epidermal tissue conjugates i.d.	Chase & Kawata (1971)[dd]
Prospective testing for human allergenicity	Review by Klecak, 1977[ee]
Guinea Pig Maximization Test	Magnusson & Kligman, 1967[ff]
Day 0, 2 sites CFA i.d., test substance in CFA, 2 sites i.d., test substance in saline, 2 i.d. sites; day 6, 5% sodium lauryl sulfate; test days 7, 21, always under occlusive dressing	Marzulli & Maguire, 1982[gg]
Modified Split-Adjuvant Technique	Maguire, 1973[q]
Shave, local dry ice; salve applied days 0, 1 under occlusive dressing; day 4, CFA at margin of area, salve days 4, 7, 9 with occlusive dressing	

[a] Reale Accademia d'Italia, Rome: Convegno Volta, 50; [b] *J. Exp. Med.* 64:625; [c] *J. Phar. & Exp. Ther.* 82:377; [d] *Dermal Toxicity*, Ass'n of Food and Drug Officials of the United States, Austin, TX: Texas State Dep't of Health; [e] *Arch. Dermat. Syphilis* 162:345; [f] *Arch. Dermat. Syphilis* 163:223; [g] *Klin. Wochenschr.* 17:1171/*Dermat. Wochenschr.* 107:1393; [h] *J. Exp. Med.* 86:489; [i] *J. Exp. Med.* 68:505; [j] *J. Exp. Med.* 72:361; [k] *Proc. Soc. Exp. Biol. & Med.* 46:223; [l] In *Mechanisms of Hypersensitivity*, ed. J. H. Shaffer, G. A. LoGrippo, M. W. Chase), 713, Boston: Little Brown & Co.; [m] *Arch. Dermat.* 91:171; [n] In *Current Concepts in Cutaneous Toxicity*, ed. V. A. Drill, P. Lazar, 25, New York: Academic; [o] *Allergic Contact Dermatitis in the Guinea Pig*, Magnusson & Kligman, Springfield: C. C. Thomas; [p] *Immunology* 14:707; [q] *J. Soc. Cosmet. Chem.* 24:151; [r] *Brit. J. Dermat.* 91:21; [s] *J. Invest. Dermat.* 48:39; [t] *Dermatologia* 48:39; [u] *Immunology* 33:305; [v] *J. Exp. Med.* 71:237; [w] *J. Exp. Med.* 73:431; [x] *Intern. Arch. Allergy & Appl. Immunol.* 5:163; [y] *Proc. Soc. Exp. Biol. & Med.* 49:548; [z] *Immunology* 2:219; [aa] *J. Exp. Med.* 115:1023; [bb] *J. Exp. Med.* 135:357; [cc] *J. Exp. Med.* 114:134; [dd] *J. Invest. Dermat.* 56:255; [ee] In *Dermatotoxicology and Pharmacology*, ed. F. Marzulli and H. I. Maibach, New York: Wiley. 305; [ff] *J. Invest. Dermat.* 49:460; [gg] *Food & Cosmetics Toxicol.* 20:67.

In my hands, the combination method worked superbly (9). Animals so sensitized reacted to contact with hapten in concentrations of 1:20,000 to 1:45,000 in triglyceride oil; the eye was markedly sensitive to the hapten; and some reactions were noted on the hairless skin behind the ears, which covers the embryonic gill clefts. The boosting probably reflects a second sensitization de novo by hapten complexed to skin proteins in the epidermal contact sites.

The frequent practice of injecting CFA with emulsified haptens or antigens into footpads deserves comment. Freund, in devising his emulsion for injection deep into the nuchal muscles, chose 0.25 mg/ml mycobacterial content in the final emulsion. But when used in footpads, the high bacillary dose, in conjunction with the appearance of tuberculin sensitization, causes breakdown of the tissues, with severe cracking and persistent sores. Many immunologists will attest to this problem, so unnecessary. The same result is attained by far smaller dosage by mixing CFA with Incomplete Freund's Adjuvant (IFA).

When CFA is chosen for sensitizing with haptens, Arthus reactivity is considerably and unnecessarily heightened relative to the degree of cell-mediated hypersensitivity (10, 11).

The newest variant among methods is the split-adjuvant technique (Table 1, p. 5). Maguire devised this by making five i.d. injections of mycobacteria in paraffin oil on day 0, then on the following day injecting aqueous solutions of DNCB into the same sites (12). The method works as well with non–covalently binding picric acid (13). Avoidance of the water-in-oil emulsion leads almost exclusively to cell-mediated hypersensitivity, with hardly any stimulus for Ig production.

Before proceeding with individual topics, I want to enter a personal note. In 1941/42, both cellular transfer of contact sensitivity and the detection of antibodies by a passive anaphylaxis procedure were discovered in the space of a few weeks. Landsteiner paid me a third and final compliment: ". . . embarras de richesses." Landsteiner died in June of 1943. I worked alone during the next year, and joined the laboratory of René J. Dubos upon his return in September 1944 from two years at Harvard. By 1956, I had moved into a new laboratory in the institute built to my exact specifications.

INHERITANCE OF SUSCEPTIBILITY FOR CONTACT-TYPE SENSITIZATION

Breeding experiments were judged useful because albino guinea pigs varied in their response to simple i.d. injections of DNCB or TNCB. These were initiated in 1936. From groups so sensitized, I mated three high responder females in turn with each of two high responder males. Progeny from the six litters were themselves subjected to sensitization. The inheritance pattern for susceptibility was indeed complex. Only a few proved to be high responders. Four of the six litters sired by the two males exhibited only moderate to poor sensitivities. The third high responder female, when mated with either male, produced highly susceptible offspring. Once the favorable parental selection was made, the colony was easily built up.

Obviously more than one pair of genes controlled the expression of susceptibility. From later and extensive experience, we reached the private conclusion that at least three gene pairs must be involved, two inherited recessively, one pair dominantly.

In time, we were able to introduce our susceptible males into the closed Rockefeller Institute albino colony after assuring their freedom from the Group C streptococcus by skin-testing with an extract of Dr. Rebecca Lancefield's strain K104. Annual, minimal sensitization with DNCB of 100 males maintained high susceptibility from this time through 1978. About two thirds of the animals proved to be suitable, but I had to buy the discards! The DNCB-susceptibility extended to most chemical allergens. Inbreeding was carefully avoided. The gene pool was broad; several other colonies were derived from the colony by selective breeding and progeny testing. Intercurrent deaths hardly occurred.

My own earlier attempts to obtain isologous stock failed, for after two years all brother-sister matings proved to be sterile, yet in Germany in the mid-1970s Dr. Egon Macher was successful. Gene deletion may have altered some characteristics.

CELLULAR TRANSFER OF CONTACT DERMATITIS AND TUBERCULIN REACTIVITY

As stated, Landsteiner was firmly convinced that cell-bound antibody was the cause of contactant sensitivity. In 1941, an all-out effort was launched to demonstrate this antibody, since it had been established that serum transfers were fruitless. The naiveté of the work represents only the state of knowledge at that time.

Extracts of "sensitive skin" were prepared from skin scrapings taken from guinea pigs at various times during the sensitizing course. The scrapings were ground with quartz sand or glycerol-saline and passed through a Carver press at 200,000 lbs/sq. in., or first defatted with diethyl ether before freezing the tissue with dry ice. Then I partially pulverized the skin in a Graeser shock press (15) by striking the cold steel plunger repeatedly with a sledgehammer. The resulting extracts were injected i.d. into normal animals. Various times were allowed for supposed fixation of antibody to the skin. Injections were made into sites pre-painted with the allergen, then post-painted. Other starting preparations of skin were obtained by five daily paintings with 2% DNCB, and both the inflamed skin and the underlying subcutaneous tissue were extracted separately.

Presumed "sensitive skin," sterilized as well as possible by Carrel-Dakin solution, was affixed to a plate of mica to keep the epithelium flat for subsequent histological examination, then painted with sterile TNCB, and

inserted intraperitoneally. Despite the apparently ideal milieu for preservation, we detected no inflammation.

Since animals sensitized by the hapten-stromata adjuvant method (Table 1, p. 4) possessed i.p. "tubercles" from the deposited mycobacteria and paraffin oil, perhaps cell-bound antibody could be freed by mild irritation of the peritoneum in the form of a moderate tuberculin shock created by injecting mycobacteria in saline. Indeed this evoked a viscid, cell-laden exudate of between 2 and 15 ml. The process had to be started near midnight, and exudates taken from near-moribund animals the next day. Clarified exudates were injected i.p. and contact tests applied to the recipients.

In the course of these futile exercises, I once transferred the sticky exudate when it was not fully clarified, but had a hint of opacity. The recipient of this became beautifully positive. The following experiment, with fully clarified fluid, was negative—and then I *knew*. When Landsteiner looked through the microscope and saw lymphocytes, he did an abrupt about-face and said, with dignity, "Yes, I thought so!" Had he suddenly recalled the work of James B. Murphy on lymphocytes, begun in 1912 and summarized in a monograph in 1926 (16)?

In the fall of 1942, many transfer experiments with exudate cells were undertaken, from guinea pigs sensitized variously—the TNP-stromata method (9), by repeated i.d. injections of the simple chemical allergens (without mycobacteria), and by skin paintings of DCNB. Ten transfer experiments were made with DNCB, eight with o-chlorobenzoyl chloride, one with phthalyl chloride. I answered a letter inquiring about the possibility of transferring DNCB sensitivity in great detail. My correspondent published *his* confirmation, but a referencing footnote in manuscript was deleted in galley.

A few trials were also made with rabbit-to-rabbit cellular transfers, with partial success since rabbits are inferior in response to chemical allergens.

It seemed appropriate to make a tuberculin test on a recipient since the donors were doubly sensitive, and naturally I did so in an early experiment. It was positive, but Landsteiner was visibly upset. He told me not to repeat such tests: "It would make the experiment too complicated."

Even while Landsteiner was living, I tried to escape from the "tuberculin shock" technique, trying Lister's Diabetic Flour and then, under Canadian wartime restrictions, its principal ingredient casein. Finally, post-Landsteiner, I tried paraffin oil injected in large volume. Peritoneal cavities of the guinea pigs were washed out with Hank's solution plus 7% normal guinea pig serum, the serum shortly being replaced with 0.25% of a non-antigenic, KOH-derived gelatin from bone. Cells from spleens and lymph nodes were teased out and could be transferred but were less effective than

the oil-induced peritoneal exudate cells. The techniques in use over many years are given in (17).

Single guinea pigs sensitized by the combination method would yield enough cells—exudate, spleen, nodes—to transfer noticeable contact sensitivity to between 12–20 recipients. All these experiments were made with our outbred stock, hence the period of transferred reactivity was limited to about a week by host-vs-graft deletion. The contact tests applied during the period of transferred sensitivity, however, caused peculiarly efficient *active* sensitization.

Two years later I resumed transfer from animals sensitized only to tuberculin with mycobacteria in oil given intramuscularly (i.m.), so that exudates were secured in a "clean" peritoneal cavity. The transferred sensitivity did not please me since the reactions to deglycerinated Old Tuberculin were not large. Jules Freund came to the laboratory on the day after we first tested some of the recipients. As we went to the Animal House I apologized for the small reactions he would see. My expert took one look at my small reaction and said, quietly and thoughtfully, "Yes, it is so," meaning his acceptance of a tuberculin reaction on a guinea pig that had not received a mycobacterial injection. When I published (18) it seemed that hardly any others would confirm me, that I would spend months or more in defense. Curiously, about seven papers appeared in confirmation, a fact that made me wonder again about my colleagues' technique.

Dubos, in whose laboratory I worked a year after Landsteiner's death, urged me to try cellular transfer of tuberculin shock. A first experiment was highly promising. Then I could not repeat the effect, so I dropped it. The demonstration came from Kirchheimer and Weiser (19), who dared to sensitize and then inject living BCG i.p. to step up the sensitivity before harvesting peritoneal exudate cells. In retrospect, the demonstration would probably have been simple if I had used guinea pigs sensitized by i.p. injections of mycobacteria and had tried the old mycobacterial shock system. So there are various ways of evaluating techniques.

CUTANEOUS ANAPHYLAXIS

A patient with an immediate reaction to phthalic anhydride had been reported by Kern; this led Jacobs et al. to sensitize guinea pigs with anhydrides, notably citraconic- (α-methyl maleyl) anhydride. Scratch testing through the chemical would cause immediate reactions in perhaps 10% of animals, and often also cause flaring at previous injection sites (20, 21). In 1937, Landsteiner and I detected anaphylactic antibodies in guinea pigs sensitized with picryl chloride; further study was warranted. Indeed

with adequate sensitization, either by direct i.d. injections or i.d. adsorbed on alumina cream, guinea pigs demonstrated antibodies transferring reactivity of so-called "early type." The guinea pigs were sensitized with acyl chlorides, acid anhydrides, picryl chloride (TNCB), 2:4 DNCB, and also with guinea pig antiragweed and antiprotein sera. (An extended paper on this appeared in 1947 (22).) Sera of one specificity (0.15 ml, either neat or diluted) were injected i.d. and tests made one or two days later without use of albumin-binding dyes. If the chemical was highly reactive, solutions were injected s.c. in triglyceride oil. Reactions would start within 5 to 45 minutes, first visible as pink points which rapidly coalesced and became edematous. The less reactive TNCB would have latent periods of one to three hours. Very large local reactions were not uncommon—44 × 30 mm, 70 × 34, 75 × 48; during the next 7–9 days, such areas would not support reactions of another specificity. But when hapten-protein conjugates were injected instead of the simple chemical, there was no delay in initiation of the reaction.

The antibodies withstood 56°C for 30 minutes, then with further heat declined slowly. Prepared skin sites remained reactive but decreasingly so for five or six days only. Just one antiserum, to ortho-chlorobenzoyl chloride, remained fixed in the skin for 13 to 20 days; surely this was IgE, but we found no similar specimens.

Recipients of washed peritoneal exudate cells (paraffin-oil induced) were screened for skin-sensitizing antibody since the donors showed intense contact sensitiveness and possessed both IgG_1 and IgG_2 antibodies. We never detected anaphylactic antibody in the recipients of exudate cells. Then a visit from Howard C. Hopps stimulated further effort; to him, my negative finding was highly improbable. It was indeed possible to detect skin-sensitizing antibody after cells teased out directly from lymph nodes or spleens were transferred (23). Apparently lymphocytes capable of accumulating in the peritoneum did not possess the cells we now know as B-cells.

The antibodies were first detected in uterine horns taken from cell recipients, tested in a Schultz-Dale bath with TNP-protein conjugates. When we excised horns at different times, we first found Ab after 2–3 days; the horns became increasingly reactive through the sixth or seventh days. This laborious technique was replaced by sequential ear bleedings of recipients tested by the cutaneous anaphylaxis method. To improve sensitivity of the test, independently of the work of Ovary (24, 25), we injected the antigen i.v., along with McMaster's purified Pontamine Sky Blue 6B. The dyestuff revealed reactions quickly, but it, and later Evans Blue, were found to yield some horseshoe-shaped reactions, which without dye had always appeared as evenly elevated.

We showed that the guinea pig anaphylactic antibody, later to be recognized as non-IgG_2 by Benacerraf et al. (26), required a latent period in skin of 17 to 24 hours for maximal reactivity, far longer than the 4–7 hours needed with use of rabbit immune serum tested on guinea pigs.

Cellular Synthesis of Antibody

In these experiments with teased-out cells, we did not find antibody within the cells, hence there had to be synthesis in vivo. Another useful model to demonstrate synthesis de novo was provided by cells taken during immunization with diphtheria toxoid. Jerne had just published studies on the stepwise increase in avidity (affinity) by guinea pigs during such immunization and had developed two methods for measuring avidity (27). Early guinea pig antitoxin possessed low avidity; a further injection led to antitoxin of moderate avidity; a final boost after resting yielded quite avid antibody. Accordingly, cells were collected at each of the three stages and transferred. Serums from both donors and recipients were measured for avidity by one of the Jerne methods (28, 29). Each stage produced antitoxins of successively higher avidity, precisely the avidity of surviving members of each donor group. Synthesis de novo clearly occurred. Others with quite different methods, particularly the use of tagged amino acids, were also proving the same now fundamental principle.

Titration of Guinea Pig PCA Antibody

A better method was needed to titer the anaphylactic antibody, once called 7Sγ1, now IgG_1, better than the typical method of injecting many dilutions of a single serum into one recipient and finding, upon challenge with antigen, the dilution-to-extinction titer. Since individual animals differ with respect to accepting PCA antibody, several guinea pigs were required for deciding the titer of every serum.

The problem was solved by selecting one standard serum of each specificity. We carefully tested every standard serum on a number of guinea pigs to determine the average extinction titer. This titer multiplied by 12 produced challenge reactions of 15 to 19 mm on different guinea pigs. Whatever the size of the standard, it was assigned a four-plus (++++) value, the result of injecting one U_{12}/0.1 ml. Other serums of the same specificity were diluted to give the equivalent reaction. With sequential use of 3 recipients, the unitage could be found on 9 or 10 sera. The first round of dilutions of unknown serums was made with dilutions expected to give *smaller* reactions than the standard. More appropriate dilutions were made for the second recipient, often leading to assigning a titer for a few sera. More appropriate concentrations were employed on the third animal and usually titers could be found for all (30).

TOLERANCE

In April 1942, we injected a few guinea pigs i.p. with citraconyl-guinea pig erythrocyte stromata before making i.d. sensitizing applications of citraconic (α-methyl maleyl) anhydride. In subsequent scratch tests with this compound, they did not react, a surprising fact. The finding clearly opened a new line of inquiry. Landsteiner was quite put out by the experiment, undertaken without prior consultation, but he at once attempted, unsuccessfully, to apply it in his war research studies with John Kidd on sensitization to mustard gas in guinea pigs.

In October 1943, following Landsteiner's death in June, I resumed work on this "unresponsiveness." It was *not* possible to repeat usefully my initial observation with i.p. citraconyl- or DNP-stromata. That outcome probably reflected inclusion of some animals among our outbred stock which by chance happened to be highly susceptible for developing tolerance. Various alternate modes of applying chemical allergens were tried. Distinct effects were observed with applications of tiny doses to the surface of the skin. In particular, three guinea pigs which had served as normal irritancy controls during the testing of a sensitized group were assigned to a group just about to be sensitized with the same material, TNCB. Only these three failed to develop sensitivity. But results along this line proved too inconstant.

During these experiments, I sought to develop an animal model of drug sensitivity per os, and had a technician feed a guinea pig gelatin capsules containing dry DNCB and lactose while the incisors were held open by a brass wedge. I also specified that this one guinea pig should have small patches of hair removed once or twice a week to see if skin eruptions were present. My assistant fed the capsules religiously but left the clippers in the laboratory. Five or six weeks later we visited the animal house, this time *with* the clippers. The skin showed nothing. I transferred the animal to a new group to be sensitized to DNCB. All became sensitive except this one animal. By sheer luck the goal of rendering almost all animals significantly unresponsive had been discovered in this, the seventeenth trial of varied methods over five months.

Since feeding capsules was laborious, we dissolved DNCB in olive oil to be fed from a fire-polished pipette, keeping the lip from contamination. The minimal number of feedings was ascertained, and experiments employing twelve to sixteen animals, half to be fed, all to be sensitized together, gave clear-cut results on unresponsiveness. Results came quickly. The tolerance was specific for the chemical used and it lasted for more than 214 days. Not only was contact sensitivity affected; anaphylactic antibodies (IgG_1), which usually appeared during sensitization to picryl chloride (TNCB), were

seldom detectable. Further, injections of TNP-guinea pig proteins into normal and pre-fed animals proved that only the naive group developed anaphylaxis upon i.v. injection of TNP-proteins.

When given white cells from contact-sensitive individuals, animals pre-fed with TNCB would respond splendidly to contact testing and then relapse into negativity as the host-vs-graft rejection ended the life of the transferred cells. (With *normal* recipients, simple contact testing following transfer of effective cells induced permanent sensitivity.) As I noted, the efficiency of the oral route of administration was high, although partial degrees of tolerance were met in animals given i.v. injection or skin painting with weak concentrations. There is no need to carry the story further, for it is covered by a recent review (31) with bibliography. I myself owe much to others, in particular Drs. J. R. Battisto and Roy E. Ritts.

Although I did not know it in 1943, Wilhelm Frei in Breslau had observed tolerance to neoarsphenamine in syphilitic men who had received i.v. injections for therapy. Unlike normal or new patients who became sensitized by the first i.d. dose, these men would not respond to sequential i.d. injections of the drug. Frei also demonstrated that guinea pigs could be sensitized by a single i.d. dose. Dr. Marion Sulzberger, then in Breslau, extended the experiment to guinea pigs (32). He injected guinea pigs i.d. with 150 μg neosalvarsan, and on the next day injected six of them i.v. with one-third of a lethal dose of the same chemical. These six failed to respond to a subsequent i.d. test, while the remaining animals became positive. Returning to this country, Sulzberger found that he could not sensitize guinea pigs with neosalvarsan and his observation was not followed up. Some years later I resumed the subject, altering the sensitizing procedures and feeding the compound in small amounts rather than duplicating the large i.v. dose. Thus the same principle did indeed link our battery of chemical allergens with neoarsphenamine and Old Salvarsan (33).

I had become convinced that long-term tolerance to TNCB was maintained by the presence of TNP groups anchored to tissues—in hindsight a ridiculous proposition. One experiment should have opened my eyes; it did not. By switching from outbred guinea pigs to Wright's Family XIII inbred animals, and then carrying out further brother-sister matings for a few years, we finally obtained fairly long-lasting cellular transfer, for 68–110 days. Here, cells from sensitized animals would transfer the contact-sensitivity to TNCB satisfactorily to normal animals but *never* to animals made tolerant to the chemical beforehand. Evidently a special restraining mechanism was at play in these animals to thwart cellular transfer. I did not see that, nor did I take a fresh look on the mechanism of tolerance when

Battisto and Bloom were able to secure unresponsiveness in 79% of guinea pigs by giving one i.v. injection of TNP-coupled spleen cells or TNP-homologous erythrocyte stromata. Their experiment showed that the tolerizing antigen need "not reach all potentially immunocompetent cells" (34).

Six years later, Gershon et al. discovered suppressor cells (35), a fact which revolutionized in vivo experimentation in immunology.

PENICILLIN AS SENSITIZER

The early production of the precious penicillin (from growths in two-quart milk bottles) was reserved for Army use, where however some problems had arisen with soldiers. Walther F. Goebel and I went to Boston to see Chester Keefer, in charge of Army records and the distribution of penicillin. We were shown a few case records; I noted that allergic reactivity usually occurred following one course of treatment in hospital, a rest period, then resumption of treatment. Keefer was convinced that the problem had to do with impurities in the preparations. I was to attempt to sensitize guinea pigs. One vial of 60,000 units would be delivered at a time; this would allow use of 6 animals. My plan was to apply the substance in a mixed solvent repeatedly on areas of skin lightly irritated by dilute cantharidin to provoke delayed-type sensitivity rather than to search for immunoglobulin production. As new vials were called for, the weight of lyophilized material containing 60,000 units dropped steadily while the color lightened, as better fractionation methods were employed. Two guinea pigs became sensitive, one quite satisfactorily, the other moderately. Crystalline penicillin had just been introduced, after the chance discovery in industry's new tank cultures of *P. notatum* that lyophilization from *toluol* would produce the crystals so long sought. I requested enough to test my guinea pigs. The request was denied. Shortly, Welch and Rostenberg used the crystalline product to demonstrate sensitivity of tuberculin type in a botanist who had long cultured *Penicillium* spp. but had no contact with penicillin itself (36). Thus its sensitizing ability was demonstrated, and Chester Keefer had his answer. It was Bernard Levine, skilled in chemistry and medicine, who succeeded in sensitizing guinea pigs regularly by i.d. injections and in showing the role of penicillin degradation products as well (37). Cell-mediated hypersensitivity arose when penicillin was used as a "strewing powder" on the cracked-open feet of soldiers in the Tobruk desert. Most manifestations of hypersensitivity were related to IgE antibody, as in the Army hospitals and later in the use of penicillin lozenges. Penicillin was not to be condemned, but we had to use it more intelligently.

THE SALK POLIOMYELITIS VACCINE*

On a Friday morning in mid-1954, René J. Dubos charged into my laboratory and announced that I must go *immediately* to Detroit; he described this as an order from Tom Rivers, then Acting Director of the RIMR. The Salk vaccine just produced at Parke-Davis was under a cloud, for when injected into a tuberculin-sensitive guinea pig it had given a reaction. The vaccine must contain tuberculin from mycobacterial-contaminated monolayers of monkey kidney cells used to support the multiplication of polio virus.

After a moment I said quietly, "I shall not go. What type of animals would I find there, what assurance of their history? My own sensitized guinea pigs are here, and my equipment. The only question is whether a product contains tuberculin."

Dubos returned shortly and said that Dr. Henry Kumm was leaving for Detroit straightaway on Basil O'Connor's directions, and would return with samples around 11 pm that evening. The donor monkeys had passed their tuberculin testing, and it was highly unlikely that any effective "tuberculin" could be produced during the few hours on the monkey kidney monolayers.

Waiting when Kumm arrived were the animal cages brought to the lab, animals boards, clippers, necessary diluents and, of course, tuberculin. I believe that there were three samples. Dr. Kumm's eyes followed every move as I made out the protocol, checked ear tags, loaded and labeled syringes. All the while my courier perched on a laboratory stool for two hours, watching but saying nothing. The cages went back to the animal house, and I went to New Jersey.

In those days Saturday mornings were spent at the laboratory. But I did not appear. At least 11 hours had to elapse before a first appraisal of reactivity to tuberculin would be possible. And Rivers would be waiting for me at 9 am! He appeared in Dubos' office at 9:30: Dubos could not explain my absence. At high noon I arrived and went directly to the animal house. All the animals, controls and sensitives alike, showed queer reactions; true tuberculin reactions had developed only on the sensitized guinea pigs. Next, I went to the lunch room to meet Rivers, who charged over at once: "Well?"—"Their report was a fake." Rivers said no more, but perked up visibly as he regained his table.

Repetition of the tests showed that the reactions of the Parke-Davis samples were of chemical origin, developing within a few minutes and

* Rivers' account appears in reference 38.

shortly reaching their maximum. Had I stayed another hour the night before, the nature of the problem would have been apparent then. It was incredible that a commercial laboratory had failed to inject normal animals as well, and did not know what real tuberculin reactions looked like.

The most obvious substance to test was Merthiolate (thimerosal) at 1:10000 strength since it was prescribed for the final packaging. The chemical duplicated the reactions exactly, as did additional samples of the compound. Few persons seem to know that Merthiolate-preserved fluids are toxic for guinea pig skin; rabbits do not react similarly. Rollin Hotchkiss sketched out an isolation procedure for identification, which I followed. All the Detroit samples were positive. Then a telephone call came from Detroit, from three executives on individual phones, whom I shall not name. In unison they announced that I was wrong, there had been *no* merthiolate in the samples taken to New York. It was futile and curious. Their production run was no longer under a cloud, they had never tested normal guinea pigs. Wearily I stated that chemical identification of Merthiolate had been made, and hung up the phone.

THE ADJUVANT AND SENSITIZING POTENTIALS OF MYCOBACTERIA

Further Breeding Experiments

Now so uniform to sensitization with simple chemical allergens, our stock varied in sensitization with TNP-stromata plus mycobacteria (8) and in the extent of boosting in the combination method (9), as determined by contact tests with TNCB, and also in the degree of sensitivity acquired to the tuberculin.

To test the *adjuvant* effect of mycobacteria, colonies of high and low responders were derived from our stock (TNP-stromata plus mycobacteria, tested with TNCB). In parallel fashion, we set up high and low responders to tuberculin. Members of the four colonies were sensitized at the same time to see which would respond in fundamentally different fashion. In a small but significant proportion of the animals, we found that the *adjuvant* and *tuberculin-sensitizing* capacities of mycobacterial cells reflected the participation of different bacterial constituents, as Raffel had once reported (39). During these tests, a new phenomenon came to light, which I now will discuss.

Multiple Disseminated Granulomata

The four colonies just mentioned had been injected with TNP-stromata and with citraconyl- or maleyl-stromata in our type of CFA, to allow initial

tests for contact reactivity to the two haptens, then a test to determine the effect of any boosting through the repeated skin tests, and finally a tuberculin test. Repeated clipping of hair was necessary, during which my technician observed "cords of tissue," firm to the touch, extending out from older contact sites. Eventually we learned that, although all injections had been into the nuchal muscles, the initial lesion started spontaneously in paragenital position, three times as frequently in females as in males. Fairly frequently, secondary swelling appeared around an ankle, a pale image indeed of the pronounced adjuvant arthritis in rats (40). The striking feature, however, was a progressive development of multiple disseminated granulomata on the flank at aging contact sites or tuberculin-sites. Alternatively, sites irritated by cantharidin or places along old surgical cuts through the dermis were foci for inducing nodules. The first tiny nodules appeared within 5 to 21 days after a test, after which extensions along but over skin lymphatics developed for another 15 days (41).

The animals most prone to genital lesions came from two colonies: the high tuberculin reactors, and the animals which *failed* to utilize mycobacteria well as *adjuvant*. The nodules were epithelioid sheets developing along but external to lymph channels. Epithelioid sheets in the paragenital region lay in connective tissue around collections of fat cells.

The underlying factors for developing these disseminated lesions were: (*a*) selective genetic background, particularly among females; (*b*) high sensitivity to tuberculin; (*c*) a primary development of paragenital nodules, after which specific or non-specific stimuli could cause translocation of nodules to sites of dermal insults. We may presume that macrophages containing some bacillary remnants, plus lymphoid cells already "primed" to recognize mycobacterial cell walls arrived at intervals in the sites of dermal insults.

There are resemblances to the dermal lesions of sarcoidosis, which are often to be detected in old scars, and are called forth by injecting i.d. the insoluble "Kveim-type" material from sarcoidal spleens. Not in detail, but perhaps in principle, the possibility of antigen-bearing cells or actual antigen-deposition in skin encountering wandering "trained" lymphocytes may be common both to these nodules and to dermal lesions of sarcoidosis. In sarcoidosis the antigen remains unknown and probably is different from the tubercle bacillus. The characteristic bilateral hilar lymphadenopathy of sarcoidosis is not seen in guinea pigs, yet epithelioid sheets with giant cells are present in both.

Responses of Established Lines of Inbred Guinea Pigs

When breeding of Sewell Wright's inbred Family 13 and Family 2 guinea pigs was started, we subjected them to our series of sensitization techniques.

The two colonies were inferior to our own albino stock, and they differed from one another chiefly in susceptibility to tuberculin sensitization and to the adjuvant function of CFA towards TNP-stromata. In both respects, Family 2 animals were poor responders (42).

In order to dilute out the special characteristics of Family 13, we made backcrosses to Family 2. By the sixth backcross, the animals resembled pure Family 2 stock in sensitization pattern. But the first mating of these animals to Family 13 yielded progeny exactly like Family 13 stock. Evidently there is a dominant gene pair in the 13's which enables heterozygous stock to possess the sensitization pattern of Family 13.

In later admirable experiments by the Benacerraf group on antibody production by 13's and 2's, gene pairs were discovered which exert a high degree of control. The differences came to light when tiny doses of DNP carriers were injected with CFA into 4 footpads of guinea pigs (43). Family 2 animals possess a particular gene termed PLL, the term being derived from use of poly-L-lysine (PLL) or DNP-PLL. Family 13 lacks this gene for synthesizing antibody under minimal stimulation, and hardly responds to DNP-PLL or DNP-BSA. Family 13 possesses a special gene pair which make this strain high responders to DNP-guinea pig albumin (44), not shared by Family 2's. The two strains are easily distinguished even when high doses are used if dimeric DNP-GPA is excluded (44a). Both of these gene pairs are associated with HLA antigens in the individual stocks. There is no present knowledge to suggest that the Family 13 dominant gene seen in the backcross breeding described above is the gene involved in the Family 13 response to DNP-GPA.

Additional differences between Families 13 and 2 were found by Polák et al. (44b). Only Family 13 could be sensitized to mercuric chloride, only Family 2 with potassium dichromate and beryllium fluoride.

Reports of Transfer of Tuberculin Reactivity with Plasma

Various claims have appeared, stating that true tuberculin reactions can follow injection of plasma from sensitized guinea pigs (e.g., 45, 46). In one instance (45), a special "Cohn-type" cold alcohol fraction called IV-10 was reported to effect transfer; certainly it was free from all then-known Ig's, which were to be found in Cohn Fraction II. Our method for titrating PCA serums proved especially useful in investigating the IgG_1 content of the plasmas used. When we isolated Fraction IV-10 in our laboratory, it was rich in "tuberculin-specific" IgG_1. Dupuy et al. (46) stated that X-irradiation was necessary to obtain effective plasma. Their donors had been sensitized by two successive injections of living BCG, then skin-tested, irradiated, and bled on following days 3, 4, 5. Each recipient was given 2–3 ml of fresh plasma per bleeding. When we repeated the experiment with

PCA titrations of serums taken throughout the procedure, it turned out that the *second* injection of BCG was critical as well as the tuberculin test itself (47) in raising IgG_1. X-irradiation could be omitted, although a few individuals were boosted in titer, thereby raising the unitage of IgG_1 in pooled plasma (48).

In investigating both claims, we found that successive injections of plasma pools contained large amounts of PCA antibodies, so that, upon skin testing, a nearly immediate PCA-type reaction commenced and increased for several hours as circulating IgG_1 fixed at the local tuberculin test. The pronounced edema then receded, leaving residua of 2–8 mm when readings were made only at 24–48 hrs. The respective authors had interpreted a 24 hr reaction of 5 mm to be a "positive" tuberculin test, evidently without careful screening for the early reaction.

THE END OF LIVER POWDER AS ABSORBANT

When the unstable fluorescein isocyanate was introduced by our own Albert Coons for conjugation with antibody (it was used as soon as prepared), positive fluorescence was viewed as green against a dark background. In case the background displayed fluorescence, Coons absorbed the conjugate with "liver powder." Then came the introduction of the stable isothiocyanate derivative, yet the Coons' ratio of 50 mg per gram of protein was maintained. Liver powder was used routinely, yet fluorescence was now accepted as a gold color against a background of strong green. The result was acceptable if the test object possessed a sharp outline, such as a bacterium, but there would be confusion in localizing diffusely spread materials, such as antibody in its nascent stages.

It was a rewarding moment (I myself was studying the role of liver powders) when Gerald Goldstein in my laboratory, with John Cebra's help, solved the problem (49). Fluorescein isothiocyanate at 50 mg/gm combined with too many sites on the gamma globulin molecule, thereby upsetting the zwitterion milieu of the protein and causing it to precipitate onto protein surfaces in general.

The solution was to couple the stable isothiocyanate using only 6–8 mg per gm of globulin, then to separate, on small columns of DEAE-cellulose, those molecules coupled with a suitably low number of fluorescent radicals. Later work with Cebra (50) extended the methodology. Goldstein's method was condemned at once by many workers as being "unnecessary," yet within a few years no one was using liver powder and the chromatographic separation technique was practiced widely. Neither liver powder nor Goldstein appear in the index of a 1983 volume on immunofluorescence, which attests to the success of his endeavor.

NIH COMMITTEE ON THE STANDARDIZATION OF ALLERGENS

Several advisors to NIH, cognizant of the success of Spies and Coulson (51) in separating peanut and castor bean allergens, urged that purified components of allergenic pollens might be more effective for treating patients. In June 1959 under the chairmanship of Dan H. Campbell a large group—allergists, immunologists, a botanist, representatives from firms preparing allergenic extracts, officials of NIH—assembled to plan approaches. The first project was to be short ragweed. As a uniform source for individual scientists who might be interested, pollen would be purchased in quantity, then dried, drummed, and packaged by a commercial firm.

Shortly, Campbell set up three subcommittees. As chairman of the Subcommittee on Biochemistry and Biophysical Separation of Ragweed, I funneled requests for pollen to Campbell and with his approval to the repository of NIAID.

Studies on ragweed were then in progress in about eight laboratories. The existance of about 13 antigens were known, but not which of them might be allergenic (52–54). I proposed assembling all workers on ragweed to present the current status of their work. In April 1960 I set up a Conference on Standardization of Ragweed Pollen at The Rockefeller Institute. All invitees accepted, including Bram Rose's Subcommittee on Immunological Testing and William B. Sherman's Subcommittee on Skin Testing, and NIH personnel. Eight major papers were presented and discussed over two days. Technical procedures were seen to vary from one laboratory to another, while the various chromatographic runs located "active" fractions in different positions in the effluents. Following the discussion, the workers disbanded, now to use the standard pollen.

The first single antigenic component of ragweed was obtained in 1962 by T. P. King of Lyman Craig's group at The Rockefeller Institute. Termed Antigen E (Ag-E), it was found to be highly skin-reactive in patients (55, 56). Despite my pleading, other antigens were not sought for some time. Indeed the number of laboratories working with ragweed became fewer.

A commercial source for preparation of Ag-E was located, and samples soon arrived for testing in vitro. Shortly, some of this uniquely pure antigen became available to Dr. Ishizaka in Denver. Upon radioiodination, Ag-E allowed proof that concentrated reaginic antibody would precipitate Ag-E but only if the patient was indeed skin-reactive to Ag-E (57, 58). The torturous story of how human reagin was identified with a new immunoglobulin, IgE, is given in reference 59.

The first batch of Ag-E was consumed in therapeutic trials, and then a second lot, but AgE did not prove to be particularly effective for treatment.

Yet as pure material the Ag-E served uniquely for in vitro tests, and its yield from fresh pollen is nearly constant, so that its concentration in commercial aqueous extracts serves to monitor their quality. To secure more Age-E, NIH advertised for bids, and time was spent in evaluating the competence of responding firms. The experience with such firms was disillusioning. Several firms in turn spoiled good pollen by deviating from the carefully prescribed protocol. University laboratories, put aside since they would have needed extra equipment, would have been more efficient and probably less costly overall.

The Chairman's Grant to Dan H. Campbell finally proved embarrassing to NIH, for it had been renewed beyond precedent. The day came when we all received Certificates of Grateful Appreciation of the USPHS, dated only 1798, its founding date. Yet the task of purifying allergens, as my return letter to Dorland Davis pointed out, had hardly begun, despite the fact that "allergy" is included in NIAID. The task was resumed later on, fortunately, with a different mechanism. It is worth recalling that other ragweed antigens were purified—Ag-K (King), Ra-1, Ra-2, and so on. Indeed, some of the smallest molecules, to which very few persons possess a clinical allergy, are found to sensitize individuals of particular HLA type.

SARCOIDOSIS AND THE KVEIM TEST

With the establishment of a sarcoidosis clinic at The Rockefeller Institute Hospital in 1957, I was eager to watch the intradermal reaction to Kveim test material, prepared from heated suspensions of human sarcoidal tissues, preferably the spleen. Within four to eight weeks a local granuloma would be palpable at the injection site and be confirmed histologically by biopsy. Only active cases of sarcoidosis would respond in this way. I was to prepare the test material. After some initial experiences with autologous lymph node materials, we turned to the spleen "Johnson" of Dr. Louis Siltzbach; it was well validated for potency, absence of tissue-irritative substances, and freedom from transmissible agents. The furnished preparation was a crude suspension of tissue and coagulated hemoglobin. The usual test dose, 0.15 ml, contained 1.1 mg by dry weight.

It was not difficult with splenic material to make several improvements, starting with washed subcellular material and with strict sterile technique. The average test dose was at once reduced to 450 μg (60), while a later product was active in less than 50 μg (61). Still smaller doses showed dose-dependent reactivity. The preparation was stable to acid, but rapidly labile to alkali.

During each and every step of manipulation, Siltzbach would say, "somewhat weakened in potency." He said this invariably whether three or

seven intervening steps had been used, hence his complaints were not additive. Finally I devised a trap, which surprisingly was accepted. Selected patients would each be tested with three materials from vials bearing only the patient's name and a number. The preparations would differ in concentration and be permuted among test sites. Included was one duplicate pair of vials for each patient. When biopsies were finally in hand, Siltzbach was taken aback—ratings between duplicated tests, although always positive, varied markedly, for particulates could *not* be deposited precisely in the same way in the dermis.

Kveim tests with this validated source material proved to be highly useful (62), positive reactions accompanying X-ray evidence of activity. For the few patients seen within three or four days after the test injection, I was able to predict future biopsy-positive reactions from early swelling and local palpation, even though four or more weeks would be required for organization of the "diagnostic" granulomatous sheet which pathologists required for acceptance of a positive test. One or two patients who visited the clinic when their disease was inactive, and therefore did not undergo local biopsy, returned to the clinic months later with a newly positive nodule at the previous injection site accompanying reactivation of bilateral hilar nodes.

Many liters were prepared from this spleen, which was vialled commercially and sent worldwide, with biopsy sections returned for confirmation here. Then Federal authorities stopped interstate transportation of preparations made from human tissue. A few larger hospitals did prepare their own test materials from available spleens—under conditions questionable for safe validation of their suspensions.

Contracts were shortly let by NHLBI for development of an in vitro test as substitute for intradermal testing, but none of the several workers (as I anticipated) was successful.

MECHANISM OF SENSITIZATION

Injection of Simple Hapten without Adjuvant

The nearly obligatory role of intradermal injections in effecting contact-type sensitivity was noted early, but was explained most clearly by Macher (63). Radiolabelled TNCB or DNCB was injected into the tip of one ear in a dose capable of sensitizing two-thirds of the guinea pigs. By timed ear excisions and combustions of the injected sites within sealed vials, Macher learned the rate of loss of chemical from the ear. After ten days, the one-eared animals were given contact-tests to determine if sensitization had occurred, and if not an active sensitizing procedure was applied. With TNCB, the ear site was needed for at least 48 hours, when only 7% (20 ng)

remained; but 4 days were required for full sensitizing potential. DNCB required a larger dose, ca. 3.5 μg, because of its faster escape from the ear depot; the ear site was needed for 24–36 hrs, at which time 30 to 40 ng remained in the ear.

Animals not developing sensitivity because of early ear-excision were given an active sensitizing course, which revealed that many had acquired significant degrees of unresponsiveness (tolerance).

Loss of allergen occurred through walls of blood vessels serving the ear, not as we had expected by passage into lymphatics. Passage of allergen into the blood stream resulted in a discernible degree of tolerance, which could be duplicated by i.v. injection of the equivalent amount into normal guinea pigs. The conclusion was inescapable that the degree of sensitivity which arises by i.d. injections of allergenic chemicals is the balance struck between sensitization and tolerizing effect, that is, sensitivity is a resultant of the relative abundance of effector and suppressor cells (63).

Accordingly, sensitization to haptens by i.d. injection is, to use Medawar's phrase for homograft rejection, "peripheral," the lymphoid cells encounter the hapten-fixed tissue at the peripheral injection site, rather than centrally within the lymphatic organs. Cells experiencing the fixed hapten enter the lymphatics and multiply within nodes. Peripheral sensitization is also probably the mechanism when sensitizing contact applications are made to the epithelia of vagina, uterus, and colon (64).

In repeating Macher's excision-of-depot experiment, but with application of a relatively high dose of pentadecylcatechol (ca. 0.9 mg PDC) to the skin, Godfrey and Baer (65) found that ca. 0.3 mg PDC left the site within one hour, appearing widely in lymph nodes and internal organs, although later absorption from the skin was slow—44% remained locally at 72 hrs. Yet excision of the application site at 24 hr diminished notably the full expression seen in the "positive controls." The special solubility of PDC, a stable derivative of poison ivy urushiol, and the size of the dose were less discriminating than the conditions of the Macher experiment.

Hapten-carrier Conjugates as Active Sensitizers

To sidestep, if possible, the tolerance-factor of i.d. injections of simple chemicals, we coupled defatted, finely subdivided particles of guinea pig epidermis with TNCB and nullified any residual trace of TNCB by secondary coupling with glycylglycine. Following i.d. injection at one time, alone or with alumina, tests made on day five showed that full sensitivity would be established on day six, much more intense and a day or two earlier than in sensitizations with the chemical itself, with its tolerizing effect (66).

In contrast to these experiments with skin particles, TNP-epidermal *solutes* would function to some degree as sensitizers if adsorbed to alumina

and injected i.d. TNP-guinea pig albumin was ineffective. Salvin and Smith (10) had been able, by using the footpad route and incomplete Freund's adjuvant, to obtain contact-sensitivity in 62% of their animals with TNP-epidermal *solutes*.

Injection with Freund's Adjuvants

When sensitization is induced with materials emulsified in adjuvants, even incomplete Freund's adjuvant lacking mycobacteria (IFA), whether the material be either simple chemical or hapten-homologous skin conjugates, a great thrust for immunoglobulin synthesis starts. Thus Salvin and Smith (10) found precipitins and pronounced Arthus reactions shortly after appearance of cell-mediated hypersensitivity, as did Benacerraf and Gell (11).

If parenteral injection of aqueous solutions within CFA is used, we must posit other mechanism(s) than peripheral sensitization, since intradermal or footpad injections of the oily emulsions pass down the lymphatic pathway, with mycobacteria found widespread in oil droplets (67). Apparently "training fields" for lymphocytes must be established wherever hapten is released by macrophage ingestion of oil droplets, with secondary or primary involvement of the lymph nodes. CFA has been requisite in some sensitizations aimed at securing cell-mediated sensitivity (11), and has been used in part with catechols (68).

In the first profitable experiments on the adjuvant function of mycobacteria in paraffin oil, injections i.p. of antigen and adjuvant were made on alternating days, hence there is no absolute need for an emulsion form (8) despite its general superiority. The split-adjuvant technique of Maguire (Table 1, p. 5) employs separate injections as already mentioned, with exaggerated sensitivity to DNCB and great diminution in immunoglobulin formation (12).

While the role of CFA is not well understood, special experiments of Maguire with protein antigens injected i.d. in tiny amounts point to one possible facet. Doses of ovalbumin, given i.d. in five sites on one flank, did not lead to an effective sensitivity on day seven (testing on day six). Yet when Maguire injected CFA into the same sites 3 days *after* the oralbumin injection, excellent sensitivity was found. The suggestion is made that the CFA enters the draining nodes, stimulating a selective multiplication of the few cells there which have become committed (69).

SENSITIZATION WITH NON-COVALENT BINDING PICRIC ACID

Certain novel features have emerged from experiments on sensitization with picric acid (PA), which is to be secured regularly by the split-adjuvant

technique (Table 1, p. 5), but not by incorporating picric acid in CFA. Just as in the Combination Method used with co-valently binding haptens (Table 1, 3.e), successive contact tests boost the sensitivity greatly.

The initial contact reactions are characterized by microvesicles in the epidermis, largely around hair follicles, with an infiltrate rich in neutrophilic polymorphonuclear leukocytes, and some eosinophilic cells, in addition to round cells. The reaction extends beyond the area of contact application. There is a slow evolution to peak reaction at about three days, leading often to a thick micaceous scale and eventual scarring, quite unlike contact reactions with couplers like DNCB, DNFB, and TNCB. With spaced contact tests, it becomes necessary to reduce the concentration from 1% to 0.06–0.006%, and peak reactions now occur by 24 hr (70).

Despite the special features, sensitivity is readily transferred with oil-induced peritoneal exudate cells, and with cells from spleen and nodes. Cell transfers from first-test animals reproduce the slowly developing reaction, whereas cells from donors tested several times give 24-hr peak responses. The 24-hr maximal response persists even if fewer cells are transferred or a reduced test concentration is employed. A *qualitative* change, then, occurs in the lymphoid cells during the boosting process.

Animals made tolerant to PA by feeding do not sensitize either with PA or TNCB, but TNCB-tolerant animals, inert to TNCB, divide in their ability to respond to PA—some respond, others do not. Flexibility at the oxygen atom of PA appears to permit the planar 2:4:6 trinitrophenoxide radical to fix to hydrophobic patches of proteins by hydrogen bonding (discussion in 71).

In sufficient concentration, acidic PA precipitates proteins by combining with their basic groups. When *PA-precipitated* proteins are injected i.d. on several occasions, a mild tuberculin-type reactivity arises to i.d. PA, which is transferable with lymphoid cells in complete absence of *contact* responses to PA. This is a clear instance, although rare, that intradermal and contact reactivity to the same hapten can be separable phenomena. And animals injected i.d. with PA-precipitated proteins produce a unique form of IgG_1 (71).

MYCOBACTERIAL ANTIGENS

Final investigations dealt with soluble antigens of *Mycobacterium tuberculosis*, with a new approach—finding guinea pig IgG_1 antisera specific for particular antigens. We could finally identify 14 antigens. To apply a PCA technique, it was necessary to invert the usual procedure, in which known antigens seek antibodies in serums, and instead to lay down standard antiserum sites to determine what antigens would be present in column fractions. Dilutions of these serums were standardized to give $1U_{12}$/0.1 ml,

and nine such sites could be placed on each recipient guinea pig. We had to place one further site with a standard serum of another specificity to ascertain the readiness of the recipient to give PCA reactions. A column fraction of given protein content mixed with the control antigen was injected i.v. at 17–24 hr. The reaction to the standard control system was rated four-plus, and reactions at experimental sites were rated in comparison with the control site. Thus we could determine the antigens present and their relative amount. In this work, we succeeded in identifying one antigen peculiar to *M. tuberculosis* and to *M. bovis*, present both in raw culture filtrate and in the heated product "Purified Protein Derivative" (PPD), but which was lacking in the so-called "atypical mycobacteria." Previously it had been thought that no distinctive antigen existed. We hope this observation can be exploited. We found also a simple way to increase the specificity of Seibert-type PPD preparations through Sepharose chromatography, followed by rejecting the early-eluting carbohydrate-rich material (common to all mycobacteria) and also the late-eluting smaller molecules (having little skin-reactivity). Perhaps this observation will not be forgotten.

CONCLUSION

In the foregoing, I have outlined the present state of knowledge on direct sensitization with simple chemical allergens. For such studies, guinea pigs are most suitable, provided that a susceptible strain is available. For such work with haptens, inbred families 13 and 2 are only marginally useful. Guinea pigs can become exquisitely sensitive to contact testing, and repeated tests and titrations of sensitivity can be made. While studies on mice are possible with *strong* sensitizers, large numbers must be employed to offset the inadequacy of contact-testing of one ear and one hind foot, the opposite members serving as controls on swelling. Erythematous clues are negligible. Yet there is a wide choice among isologous mouse strains. Other species, too—the hog, rat, hamster, cat, chicken, cattle—have been sensitized with DNCB.

I shall end with some thoughts about the milieu of research. In my early years, colleges and universities furnished supplies for their graduate students—and part-time jobs to pay tuition. Equipment was simple indeed. The Rockefeller Institute for Medical Research stood nearly alone with its endowed facilities and a staff pledged to full-time service. The intervention of government in the support of science still looms large to me. The explosive increase in the size of *Federation Proceedings* after 1950 is a testimonial to this funding. With the new source for equipment and supplies, and then in halting fashion for salaries, commercial improvement of equipment began and the process was speeded by the concurrent

electronic revolution. But various rulings over the years have increased costs of research and of animals—and also the number of unfunded Grant applications. The granting apparatus as presently employed is unsuited to our real needs. My early experience at the Rockefeller Institute proved the value of freedom in pursuit of a problem, which included exploration of the new and the novel, with the opportunity to change course when warranted. In contrast to our present system, where we write applications frequently and guess the course of progress over many months, a worker in Japan, although a grant is not large, knows that he can pursue his study for at least 10 years. A new way is needed for extending governmental extramural support to our companions in zealous research.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 31–58

MECHANISM OF LYMPHOCYTE–MEDIATED CYTOTOXICITY[1]

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INTRODUCTION

Studies in many laboratories over the past 20 years have revealed that there are two well-characterized classes of lymphocytes that have cytotoxic activity: the cytotoxic T lymphocytes (CTL) and the large granular lymphocytes (LGL), which are responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) and for natural killer (NK). Most studies of cytotoxic mechanisms have focused on one or the other of these distinct cell types, which have clear differences in the way they recognize target cells. In my judgment, however, the results of studies of both types of lymphocyte-mediated cytotoxicity indicate that they share basic features, which will be described in this review. In particular, three recent developments have helped stimulate the view that LGL and CTL share a basically similar lytic mechanism: (*a*) Various lines of evidence indicate that after target-cell binding is complete, the NK lytic process is similar to that previously demonstrated for CTL. (*b*) Researchers, in several laboratories, have found that cloned CTL can develop an NK-like pattern of target recognition after culture. (*c*) Isolated cytoplasmic granules from LGL and from CTL, which appear to be plausible mediators of the lytic effects of the cells, have generally similar cytolytic properties.

In this review I will survey the recent studies of events in effector and target cells that occur after the formation of a stable effector-target adhesion and will consider various possibilities for defining the nature of

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the lethal damage inflicted on the target cell. The most plausible current hypothesis for the mechanism of lymphocyte cytotoxicity is that target cell binding to a membrane receptor induces a secretory process in the effector cell in which the contents of cytoplasmic granules are released by local exocytosis between the effector cell and its bound target (1). This could be termed the granule exocytosis model. It has become more attractive with the recent demonstration of the cytolytic activities of cytoplasmic granules purified from CTL and LGL; these will be described below.

Needless to say, students of cytotoxic lymphocyte mechanisms may not all agree that NK and CTL operate by a common mechanism and that cytoplasmic granules are the source of the lethal damage. A number of recent reviews expressing other viewpoints are recommended (2–5), although their authors were not aware of the granule lytic activity when these articles were written. Other excellent, recent reviews on various aspects of lymphocyte cytotoxic mechanisms should also be consulted (6–9).

Stages of the Lytic Process

During the 1970s it became clear from work done in several laboratories that the CTL lytic process can be broken down into three basic stages, which can be studied independently: (*a*) a specific binding, which can be measured by counting individual effector-target pairs or conjugates; (*b*) the calcium-dependent programming for lysis or the lethal hit; and (*c*) killer cell independent lysis (KCIL), where the target cell dies of the lethal damage (10–12). Recent work with CTL has shed new light on the binding phase (13–15), and single cell assays of target cell lysis have provided some new insights (16), but the basic mechanism of the lethal hit remains controversial (5).

The more recent studies of the mechanism of NK-cell-mediated cytotoxicity have tended to use the techniques and concepts developed by researchers working on CTL, and it has been possible to directly compare the properties of the different stages of cytotoxicity of NK cells and CTL. Initial studies (17) using these general approaches found that the formation of NK-target conjugates, unlike CTL-target conjugates, was not inhibited by low temperature and drugs blocking energy metabolism. However, as will be discussed below, many studies now show parallels between the postbinding stages of LGL- and CTL-mediated cytotoxicity; and the NK programming and KCIL stages have been defined (18).

The Properties of Cultured Cytolytic Lymphocytes

A major recent technical advance, which holds great promise for the study of cytotoxic lymphocyte mechanisms, has been the use of IL2 and other lymphokines to allow the continuous growth and cloning of cytotoxic lymphocytes (19). Both CTL and NK cells have been cloned and can retain

the lytic specificities expected from studies of lymphocyte populations (20, 21). In some cases, such cultured cytotoxic lymphocytes have unexpected patterns of target cell specificities, including some which appear to be different from any previously described (22, 23). Most surprisingly, it has been shown that cloned CTL can become convincingly NK-like in their target cell recognition, morphology, and surface markers (24, 25). Furthermore, some cloned cytotoxic T cells have both CTL- and NK-like specificities (26, 27). While these findings do not provide compelling evidence that LGL- and CTL-mediated cytotoxicities share a common basic lytic mechanism, this is clearly the simplest hypothesis. Nevertheless, it seems possible that along with the recognition elements, some components of the postrecognition lytic pathways may differ between CTL and NK cells.

EFFECTOR CELL ANTIGENS IN POSTBINDING EVENTS

Cytotoxic T Lymphocytes

Studies of the blocking of CTL–target recognition by monoclonal antibodies have recently revealed the importance of several molecular interactions beyond those directly involving the antigen receptor. These interactions involve the Lyt 2/T8 antigens (28, 29), the L3T4/T4 antigens (30), and the LFA antigens; the latter are also involved in NK-target binding (7, 31). All of these appear to act by enhancing effector-target binding when the receptor-antigen interaction does not have a high affinity. A detailed model to explain the relationship of these interactions to the triggering of the lytic pathway is not yet available, and those studies will not be reviewed here. Other antibodies also block NK-target recognition. Antibodies blocking the lytic process at postbinding steps have not been found as frequently, but they could potentially reveal accessible components required during and after the triggering of the effector cell.

Antibodies to the human T-cell antigen known as T3 have been shown to block CTL-mediated target cell lysis at a postbinding step, but they do not inhibit effector-target adhesion (32, 33). This blocking occurred with $F(ab')_2$ or F(ab′) fragments (34) and could be overcome with Con A (33). When individual CTL clones were examined (35), only 70% were blocked by anti-T3. Resistant clones showed T3 expression and modulation comparable to the inhibitable clones, and there was no direct correlation between their ability to be blocked by anti-T3 and by antibodies to T8 and T4 (35). Leeuwenberg et al (36) discovered that anti-T3 will induce nonspecific cytotoxicity when CTL are mixed with irrelevant target cells in the presence of any of the anti-T3 antibodies, but not when mixed with antibodies against other effector-surface antigens. This effect was not observed with

normal blood lymphocytes but was found with MLR-activated CTL and cloned CTL. While many functional questions remain unanswered, these studies suggested that anti-T3 may be able to trigger the CTL lytic pathway while bypassing the normal step of specific antigen recognition.

These observations are particularly interesting in light of other recently described effects of anti-T3 on T-cell function and of the relationship of T3 to the antigen receptor. The antibody is a potent mitogen (37, 38) and stimulates lymphokine secretion (39), but these effects appear to require adherent cells (40) or a second signal provided by PMA (41). High concentrations block several T-cell functions in addition to cytotoxicity (42). Reinherz and his collaborators have provided evidence that the T3 antigen can be associated with the T-cell antigen receptor through cocapping (43), coprecipitation (44), and stoichiometric coexpression (45) of the two surface proteins. In addition to the T-cell receptor polypeptides, anti-T3 antibodies precipitate a major protein chain of 20 kd plus other bands of 20–28 kd (46, 47), several of which penetrate the lipid bilayer (48).

The above findings support the hypothesis that T3 can act as a trigger to stimulate biochemical pathways, leading to functional changes following recognition by the antigen receptor. One insight into the nature of this triggering comes from the findings of Weiss et al (49), who showed that anti-T3 antibodies can cause a three- to four-fold increase in the intracellular free calcium concentration in a T-leukemia-cell line that produces IL-2. It will be interesting to see if such calcium increases are also observed with CTL.

Researchers have shown that other antibodies block CTL function at a postbinding stage. A rat-antimouse CTL antiserum known as rat* (8, 50–52) inhibits the CTL lytic process at a postbinding and postprogramming step. The blocking antibody can be absorbed by several sources of "activated" CTL, but not by resting CTL or other lymphocytes. Unfortunately, follow-up studies have provided few insights into the nature of this inhibition. Antibodies against a lymphotoxin preparation have also been reported to block CTL function at a postbinding step (53).

Large Granular Lymphocytes

In the case of NK cells, blocking of the lytic function by antibodies is complicated by the LGL Fc receptors, which can mediate a postbinding inhibition of the lytic function (54). Nevertheless, Targan & Newman (55) showed that a monoclonal antibody against T200 inhibited the lytic activity of human NK cells against some but not most target cells. The antibody did not block conjugate formation, but it did act prior to the calcium-dependent programming stage. Other monoclonal antibodies

against T200 block NK conjugates (56). Postbinding inhibition of NK cytotoxicity has also been reported by other monoclonal antibodies against surface determinants (57, 58), xeno-antibodies against poorly defined antigens (52, 53, 59), and antigranule antibodies (see below).

EFFECTOR-CELL RESPONSES TO TARGET-CELL BINDING

Cytoplasmic Granules in Cytotoxic Lymphocytes

Granules have been observed in the cytoplasm of all types of cytotoxic lymphocytes in most electron microscopy (EM) studies. These granules are membrane bound and generally contain material that is darkly stained by osmium when seen under the electron microscope. A relatively recent advance in lymphocyte classification has been the definition of LGL, which are characterized by their low density and by the presence of cytoplasmic granules (60). In human LGL many of these granules contain striking paracrystalline tubular arrays (61, 62), which are not seen in LGL granules of other species. Researchers sometimes overlooked the fact that cytoplasmic granules have been consistently observed in CTL, starting with the earliest EM studies of CTL-target conjugates (63–66). These granules do not seem to be reliably detected by light microscopy in CTL that have been generated in vivo or in short-term cultures, but EM studies of cloned CTL have revealed striking numbers of such granules (67, 68). Grossi et al (69) recently reported an impressive correlation between the presence of cytoplasmic granules and lytic activity. They found that 15 human MLR-derived T-cell clones with CTL, NK-, or K-cell activity all contained cytoplasmic granules, while 10 other MLR-derived clones that were not cytolytic did not have granules detectable by EM. Other correlations between the quality or quantity of granules and cytotoxic capacity have been noted in studies with NK and ADCC effectors (70–73). However, in human clones having NK activity, Schneider et al (74) found that most cytolytically active cells had fewer granules than the slower growing, modestly active clones.

Histochemical studies make it clear that most CTL and LGL cytoplasmic granules contain lysosomal enzymes (75–77). The cytoplasmic granules of cytotoxic LGL leukemia cells from rats have recently been purified (78). Henkart et al found that these granules contain an extremely potent membrane-active cytolytic agent, to be described below. Although, as expected, they contained a variety of lysosomal enzymes, these apparently were not related to the cytolytic activity (79). Biochemical markers characteristic of mast cell and neutrophil granules such as histamine or neutral proteases were not present. The granules contained protein

primarily with small amounts of sugar. Five major granule proteins were seen on sodium dodecyl sulfate (SDS) gels; since their sizes (62, 58, 30, 29, and 28 kd) did not correspond to terminal complement proteins, it was argued that the lytic properties of these granules probably could not be attributed to complement (78).

Reynolds et al (80) have shown that antibodies against the purified LGL tumor granules are reactive with a number of the granule proteins and are capable of inhibiting the lytic activity of the granule cytolysin. Furthermore, they demonstrated that $F(ab')_2$ fragments of such antibodies specifically block both LGL-mediated NK activity and ADCC activity at a postbinding stage of the lytic process. As predicted by the granule exocytosis model, these results provide direct evidence that one or more granule components are required for LGL-mediated cytotoxic processes to occur, since such antibodies could not have access to the granules in the absence of secretion.

Effector Cell Cytoplasmic Rearrangement

Morphological studies of killing by CTL, NK, and K cells have revealed that the effector cell cytoplasm undergoes a pronounced rearrangement after target binding, so that most of the cytoplasmic organelles become localized between the nucleus and the target cell. Thiernesse et al (75) first observed this for CTL and showed that after incubation for 15 min at 37°, a great majority of the granules and Golgi apparatuses of effector cells in conjugates were located toward the target cell. Both of these observations were confirmed by Bykovskaja et al (65, 81), who described a time-dependent rearrangement of the CTL cytoplasm during the 30 min following target cell binding. The changes described include a reorientation of the above-mentioned organelles, a broadening of the region of membrane contact between the two cells, and an enlargement of the Golgi. Geiger et al (82) also described the asymmetric distribution of CTL cytoplasm in conjugates using immunofluorescent labeling of the cytoskeletal protein tubulin. They found that the polarized tubulin distribution is observed immediately after conjugate formation, and assumed that the initial binding of the target cell occurs preferentially via receptors asymmetrically located over the microtubule organizing center. However, immunofluorescent localization of actin in CTL conjugates (83) showed a capping-like redistribution of this cytoskeletal protein during conjugate formation; immediately afterwards, actin is preferentially localized towards the target cell, in contrast to its symmetrical distribution in free CTL. Thus it is clear that at least some of the cytoskeletal rearrangement in CTL occurs at room temperature conditions of conjugate formation.

Human K cells binding to plastic surfaces coated with Ag-Ab complexes

undergo a striking and rapid cytoplasmic rearrangement (84). This process, which requires energy and divalent ions, is characterized by a flattening and elongation of the previously spherical cells and can be inhibited by a variety of drugs. Using a flat surface to mimic a target cell allowed us to visualize cytoplasmic rearrangements including uropod formation that is not readily observed with cell targets but is suggestive of those reported in CTL (81).

In NK cells a cytoplasmic rearrangement similar to that in CTL has been described. Carpen et al (85) showed that the Golgi apparatus in human NK cells is localized between the nucleus and the bound target in NK conjugates but not when LGL were bound to nonNK targets. These authors also described a time-dependent capping-like rearrangement of the actin in 50% of the NK cells bound to good NK targets but not in NK cells bound to NK-resistant targets (86). As with CTL (83), myosin rearrangement did not occur. Kupfer et al (87) provided the further evidence for cytoplasmic rearrangement induced by target cells in a cloned mouse NK cell. Using immunofluorescence techniques, they showed that the NK cell microtubule organizing center, as well as its Golgi apparatus, becomes rearranged towards the target cell after binding.

Another feature of the effector lymphocyte–target cell interaction found with all types of cytotoxic lymphocytes is an often pronounced interdigitation of the cytoplasmic processes and membranes of the two cells (64, 75, 85, 88–90). The functional consequences of this phenomenon are unclear, but interdigitation may provide a means of maximizing the contact area between effector and target cells, corresponding to the cell spreading seen with LGL on immobilized Ig complexes.

The divalent ion requirements of the cytoplasmic rearrangements have not been well defined for any of the systems studied. If calcium is required, the cytoplasmic rearrangement would be considered part of the programming for lysis stage of the lytic process; if not, it would be considered a later part of the adhesion stage.

A Secretory Process as Part of the Lytic Mechanism

Zagury and his collaborators (91, 92) have provided morphological evidence that a secretory process is part of the cytoplasmic rearrangement in CTL. They showed that acid phosphatase, originally found in the densely staining lysosomal granules of the CTL, is deposited at effector-target junctions after the cytoplasmic rearrangement. Bykoskaja et al (65) observed the deposition of material into the intercellular space between effector and target cells. This material had osmophilic staining properties similar to the granule contents, leading to an interpretation of secretion (65, 93).

In the case of LGL-mediated cytotoxicity, there is strong morphological

evidence for a secretory process involving the cytoplasmic granules. Henkart & Henkart (1) described the fusion of NK effector cell granules with each other and the release of granule contents into the space between effector and target cells after incubation at 37° of NK conjugates. The exocytosed material contained cylindrical structures with an inner diameter of 15 nm that were previously identified on target membranes after LGL-mediated killing (see below). Podack & Dennert (94) confirmed these observations using cloned mouse NK cells. With the identification of LGL granules as the source of these structures (79), there is a complete line of morphological evidence for granule exocytosis and the transfer of secreted material to target membranes during the cytotoxic process. Other morphological evidence for a secretory process involving NK cell granules includes the observation of empty granules after the lytic process (77) and the localization of the granule enzyme aryl sulfatase at the NK-target contact region (95).

Another line of evidence for granule exocytosis in the NK lytic process has come from Neighbour et al's studies with NK effector cells pretreated with strontium. Such cells bound normally to NK targets but were unable to kill them (96). Incubation of LGL with Sr^{+2} resulted in a time-dependent loss of cytoplasmic granules, and this correlated with the loss of lytic capacity (97).

As documented in the above morphological studies, the secretory process in cytotoxic lymphocytes is unusual in that it occurs in a polarized fashion that develops rapidly with the cytoplasmic rearrangement. While many secretory processes in other cells are asymmetrical due to a permanent polarization of the cell, other examples of rapidly developing, polarized secretory processes occur in phagocytic cells (98) and in mast cells presented with a surface-bound stimulus (99).

In addition to these morphological observations, a number of other types of evidence imply the involvement of a secretory process in lymphocyte cytotoxicity. The calcium dependence of CTL cytotoxicity originally suggested this (100), since an increase in cytoplasmic free calcium appears to be required for most secretory processes triggered by membrane receptors, and this is often provided by a calcium influx (101). The demonstration that the calcium requirement occurs during the lethal hit stage of both the CTL and NK lytic process (18, 102), has lent further support for a secretory mechanism. The quantitative and qualitative aspects of the calcium requirement, as well as antagonist actions, appear similar to other secretory systems (103). It is tempting to speculate that the increase in the cytoplasmic calcium level in CTL could be provided by the T3 antigen complex, which may trigger a calcium influx in CTL as well as in the IL2 secreting cells described (49). Such a mechanism could easily account for the rapid kinetics

of the programming for lysis stage of the lytic process. The monovalent ion requirements of CTL lytic activity have been interpreted as suggesting a secretory process (104). Furthermore, Russell & Dubos (105) recently demonstrated a calcium-dependent increase in CTL potassium permeability following target cell recognition. These findings strongly suggest the existence of calcium-activated potassium channels, which are known to play a regulatory role in other secretory cells (106). However, Fukushima et al (107) did not find this type of potassium channel when they conducted patch electrode measurements of CTL membrane properties.

Studies of the inhibition of lymphocyte-mediated cytotoxicity by various drugs also supported the possibility of a secretory process, particularly at the programming for lysis phase. These include chloroquine's inhibition of CTL and NK killing (17, 108), monensin's inhibition of all types of lymphocyte cytotoxicity (25, 68, 109, 110), and the actions of inhibitors of proteases and energy metabolism which are both parallel in cytotoxicity and in mast cell degranulation (109, 111).

The demonstration of the potent lytic activity of granule cytolysins in CTL as well as LGL (described below) calls for a serious evaluation of the arguments raised for and against the presence of a secretory process in the delivery of the lethal hit by CTL and LGL. Some reports of EM studies of the NK lytic process have failed to find evidence of granule fusion with the plasma membrane (25, 67), and other EM studies of the CTL- and LGL-mediated cytolytic process have not mentioned exocytosis of granules. Such negative findings are hard to evaluate in light of the small number of granules undergoing rapid exocytosis during the lytic process (1, 92).

The lack of innocent bystander killing by CTL (112) does not argue against secretion either of an insoluble, nonspecific lytic agent such as the granule cytolysin (79) or of a soluble lytic agent with target cell specificity. Both CTL and NK cells can clearly kill multiple targets and thus are not themselves destroyed when the target cell is lethally injured (113, 114). However, studies of the action of one CTL on another show that these cells do not possess an immunity to their own lytic effects; thus the lytic process is unidirectional (115–117). While these experiments argue against some models of cytotoxicity involving the secretion of nonspecific cytolysins, this kind of model can generally be accommodated if it is postulated that the stimulus mediated by the membrane receptor triggers secretion and also a temporary resistance to the lytic substance being secreted (115). A complex series of biochemical reactions occurs during a secretory process (118), and in cytotoxic lymphocytes similar reactions are inferred from drug experiments. While it seems plausible that some of these could result in the required temporary resistance, this has not been experimentally demonstrated.

ADCC against erythrocytes was found in two systems (119, 120) to require magnesium but not calcium, which the authors interpreted as evidence against a stimulus-secretion mechanism. In the case of mouse spleen effector cells in such ADCC reactions, subsequent studies have shown that cells of the monocyte-macrophage lineage can be the major effectors (121) and that LGL-mediated NK and ADCC activity can be detected only after substantial enrichment (122). With human blood effector cells, ADCC against erythrocytes can be mediated by various effector cells, including monocytes (123), LGL (60), or non-LGL T cells (73). It seems likely that the calcium-independent ADCC against erythrocyte targets was mediated by monocytes; and the calcium-dependent ADCC against tumor targets (120), was mediated by LGL. The mechanism of monocyte-mediated ADCC against red cell targets has not been extensively investigated. However, some secretory processes do occur without calcium in the external medium (101, 124).

The mouse mutation known as beige and the human Chediak-Higashi syndrome reportedly do not affect CTL function, but they do cause defects in the postbinding steps of LGL cytolytic functions (125–128). Since these conditions are also characterized by lysosomes of abnormal appearance (129), one interpretation of these results is that the LGL mechanism involves a lysosomal granule exocytosis but that the CTL do not use this pathway. However, a defect in CTL function in beige mice has been reported (130); and since the basic defect in these mutants is unknown, a definitive argument regarding granule exocytosis cannot be made.

The studies reviewed in this section show that there is considerable morphological evidence for granule exocytosis in LGL- and CTL-mediated cytotoxicity. Such a secretory process is compatible with the ionic requirements of lymphocyte cytotoxicity and with the actions of a number of drugs. The arguments against the presence of such a secretory process in the cytotoxic mechanism do not appear to be strong.

TARGET CELL DAMAGE

Membrane Damage

The first evidence that cytotoxic lymphocytes inflict membrane damage on their target cells came from studies of the release of various markers from labeled target cells. Henney (131) found that small markers such as ^{86}Rb and ATP were released from target cells before ^{51}Cr and DNA. These results were confirmed with other small markers (132, 133) and were extended to killing by K cells (134). Martz (135) showed that the increase in ^{86}Rb permeability occurred concomitantly with the programming for lysis phase of the CTL-mediated lytic process. These results indicated early

release of small ions and suggested that colloid osmotic lysis is the mechanism of target cell death, as occurs with killing by complement (see below).

An alternative approach to demonstrating membrane damage is to study the increases in permeability among artificial or isolated membranes. My collaborators and I have used this approach with ADCC, since the recognition requirements for killing are simpler in this system than in other lymphocyte cytotoxic systems. Using electrical measurements, we found large increases in ionic permeability in artificial planar lipid bilayers under conditions where target cell lysis would occur (136). Membrane pore formation was suggested by the discrete electrical conductance increases. Several groups used liposomes as ADCC targets, but the specific marker release was either small or negative (137–139). However, substantial marker release from liposomes by CTL showing H-2 specificity has been reported (140). Such marker release required the presence of undefined proteins from human eye muscle that were incorporated into the liposomes along with the appropriate H-2 antigens. CTL apparently fail to recognize subcellular target antigens even when the appropriate antigenic determinants are recognizable by antibody (13, 14, 141). However, researchers recently reported (142) specific binding of cloned CTL to a H-2K–containing lipid monolayer. Furthermore, fusion of appropriate H-2–containing membranes into nonrecognized host target cells renders them susceptible to lysis by CTL (141, 143).

Intact natural membranes such as resealed red cell ghosts are excellent ADCC targets, and a study of the release of various sized markers by Simone & Henkart provided strong evidence for pore formation in the target membrane (144). Remarkably, the maximal pore size suggested by marker sieving was about 15 nm, or considerably larger than the complement-induced pores in these membranes (144). Dourmashkin et al's (145) examination of such target membranes using electron microscopy with negative staining revealed cylindrical structures with 15 nm internal diameters. These pore-like structures were generally similar in appearance to those made by complement, but the dimensions were clearly larger. Podack & Dennert (94) found similar structures of two sizes on target membranes after the lysis of erythrocytes by cloned NK cells in the presence of Con A. They showed that these structures are short cylinders that are stable to detergent extraction and gel filtration.

When we purified cytoplasmic granules from rat LGL tumor cells (78), we found that the granules have a potent lytic effect on a wide variety of cells (79). Granule-mediated lysis was rapid, requiring less than 5 minutes at room temperature, which potentially makes this process even more rapid than the KCIL stage of NK-mediated lysis (18). Cytolysis by granules had

the same divalent ion requirements as the CTL lethal hit ($>10^{-4}$ M calcium or $>10^{-3}$ M strontium). However, the lytic activity was not stable in the presence of calcium, decaying within minutes, and showed the heat lability and Pronase sensitivity characteristic of proteins. Although it was largely insoluble at physiological ionic strength, the lytic activity could be solubilized by high salt (79), and this soluble activity was termed a granule cytolysin. Granules isolated from a variety of noncytotoxic lymphoid cells did not have detectable cytolytic activity (79).

Purified LGL-tumor granules induced the deposition of membrane-associated cylindrical structures on target membranes in a rapid process requiring calcium, which suggests that the lytic activity was associated with pore formation (79). Evidence that LGL granule cytolysin can insert such pores into lipid bilayers came from studies with lipsosome targets (146), from which the cytolysin induced a rapid, calcium-dependent release of the hydrophilic marker, carboxyfluorescein. Electron microscopy of liposomes exposed to LGL granule cytolysin in the presence of calcium showed they contained the 15 nm cylindrical structures and that penetration of the negative stain into the liposomes was correlated with the insertion of these structures (146). These studies also showed that target-membrane proteins do not participate in pore formation.

A membrane insertion model is also compatible with studies demonstrating that a variety of lipids and lipid-like molecules inhibit LGL granule cytolysin activity (C. Yue, C. R. Reynolds, P. J. Millard, P. A. Henkart, manuscript in preparation). These results suggested that an amphipathic species is formed during the action of the cytolysin and that this species will interact with any nearby hydrophobic moiety. Electrical measurements of the action of LGL granule cytolysin on planar lipid membranes (Blumenthal et al, unpublished observations) revealed a behavior very similar to that seen with human PBL on antibody-coated bilayers (136).

In many respects, CTL appear to parallel the behavior described above for LGL during the cytotoxic process. Dennert & Podack (68) have described the deposition of two classes of cylindrical structures on the target membrane during cytolysis by cloned CTL. One class was approximately 15 nm, like that described for LGL; and a second class was smaller, with 5–7 nm inner diameters. The appearance of these structures was correlated with cytotoxicity in mixtures of different effector and target cells, and they appeared to originate from the cytoplasmic granules of the effector cell (68).

Granules isolated from cloned CTL apparently possess cytolytic properties that are generally similar to those described for LGL tumor granules (147, 148, 194). These include rapid kinetics, calcium dependence, and inactivation in the presence of calcium. As in the LGL system, the addition of

calcium to the isolated granules gives rise to the cylindrical structures seen in the EM. There are a number of similarities between these structures and those formed by polymers of the complement protein C9 (148). Granules from cloned CTL will release markers from liposomes in a rapid and calcium-dependent manner (P. Fredrickse, R. Blumenthal, J. Bluestone, P. Henkart, manuscript in preparation). The cytolytic properties of CTL granules differ in minor ways from those of LGL granules. Cytolytic activity can be detected in granules prepared from primary in vitro allostimulated spleen cells (147). Although most noncytotoxic T-cell clones do not yield lytic granules, some exceptions have been found (147); the implications of this finding are not yet clear.

At present, there is no single experiment showing unequivocally that membrane damage caused by granule cytolysins is required for target cell death to be induced by cytotoxic lymphocytes. However, the results outlined above make it clear that (*a*) LGL do induce the formation of large membrane pores in target membranes, as seen functionally and in the EM; (*b*) pore structures are assembled from the material in LGL and CTL cytoplasmic granules; (*c*) pore structures are transferred to target cell membranes during LGL- and CTL-mediated cytotoxicity; and (*d*) anti-granule antibodies that block granule cytolysin activity also block LGL-mediated cytotoxicity at a postbinding step. Antibodies to a homogeneous cytolysin preparation may provide a definitive demonstration of the role of cytolysin in lymphocyte-mediated cytotoxicity.

The Role of Colloid Osmotic Lysis in Target Cell Death

Colloid osmotic lysis occurs when the natural permeability barrier to small ions provided by the lipid bilayer becomes compromised so that a significant equilibration of small ions takes place across the membrane. The macromolecules inside the cell now exert an unbalanced osmotic pressure across the membrane, causing the membranes to rupture and release the cytoplasmic contents (149). The colloid osmotic lytic mechanism predicts an early selective increase in membrane permeability to small ions, as measured by the loss of intracellular $K+$ or other ions. As indicated above, this phenomenon is well documented for lymphocyte-mediated cytotoxicity. The most compelling evidence for this mechanism is the ability of high external concentrations of macromolecules to prevent membrane rupture by balancing the osmotic forces across the membrane after the initial increase in ionic permeability; this has been clearly demonstrated for complement-induced injury (150). Similar experiments for CTL injury to target cells pose greater difficulties because of viscosity problems. Such

inhibition has been reported (151, 152), but these results are controversial (153). Taken as a whole, the evidence in favor of colloid osmotic death for cytotoxic lymphocyte targets is suggestive but hardly definitive.

A number of studies of target cell death, principally by CTL, have challenged the hypothesis that such deaths are entirely due to colloid osmotic lysis after membrane damage. The first such studies were Sanderson's time-lapse cinematographic analyses of complement, CTL, and ADCC killing of tumor cells (154, 155). He showed that tumor targets attacked by CTL and ADCC effectors often undergo a series of dramatic cytoplasmic bulging movements termed zeiosis or membrane blebbing. Zeiosis was observed after effector-target contact but well before the loss of cytoplasmic contents made the target cells phase-dark in appearance. These observations have been subsequently confirmed in other studies of cell-mediated cytotoxicity using time-lapse cinematography. Sanderson showed that the same target cells attacked by antibody and complement did not experience zeiosis, leading to the conclusion that the mechanism of target cell death induced by cytotoxic lymphocytes must be fundamentally different from that induced by complement. Since researchers generally accept that complement acts by colloid osmotic lysis, this finding suggested that such a mechanism does not entirely explain target cell death induced by cytotoxic lymphocytes.

Further evidence that the morphological events of target cell death are distinct from those seen with complement and other toxins has been summarized by Wyllie et al (156). These morphological observations have been difficult to interpret on a mechanistic level, but they do point out that there must be differences in the mechanisms involved in various types of cell death.

Using a biochemical approach, Russell and his collaborators have clearly shown that there is a rapid hydrolysis of target cell nuclear DNA during CTL-mediated lysis. In a series of careful studies they demonstrated that this DNA breakdown did not occur when the same target cells were lysed by complement or by hypotonic lysis, but only when different types of CTL were used (157). DNA breakdown began shortly after the calcium-requiring lethal hit phase began (158). Only about 10 minutes were required to digest it to a size that could escape from the detergent-treated nucleus, although target cell lysis as measured by chromium release required a considerably longer period (159). Since these events occurred so rapidly and were not observed with agents known to cause colloid osmotic lysis, they postulated that activation of a DNAse activity in the target nucleus was involved (4). That the CTL were the source of this DNAse seemed unlikely, since it did not seem possible that enough enzyme could be delivered to the target cell interior without a cytoplasmic connection, and such connections were not

detected when careful experiments were performed (160). Thus, they proposed that CTL kill the target cell by triggering an internal disintegration process (4, 161). The rapid cleavage of target cell nuclear DNA has been demonstrated for NK and K cell cytotoxicity, in addition to that by CTL (162). The authors of this study found that the nuclear breakdown was a characteristic of mouse, but not human, target cells.

When mast cells were used as the target cells for CTL, Martz et al did not observe any prelytic degranulation or the consequent serotonin release (103). Since others had reported that these cells degranulate when exposed to antibody and complement, they argued that the lethal damage was significantly different in these two systems.

Thus, various lines of evidence indicate that membrane damage followed by colloid osmotic lysis cannot readily explain all of the observed types of target cell damage; however, they do not rule out damage to the target cell membrane as a primary cause of target cell death.

POSSIBLE MEDIATORS OF TARGET CELL DAMAGE

Granule Components

As outlined above, the current evidence for membrane damage by granule cytolysin in LGL-mediated cytotoxicity is strong. For CTL the evidence is less conclusive, since there is no reported blocking of CTL cytotoxicity by antigranule antibodies. The granule excytosis model allows for the possibility that other granule components may play a role in target cell damage after the implantation of a pore by the cytolysin. Granule lysosomal enzymes are potentially able to play this role and have been suggested as participants (17, 92, 95). However, they do not appear to cause detectable target cell death on their own (79). It would be particularly revealing if cytotoxic lymphocyte granules were shown to contain a DNAse—whether lysosomal or of some other character—and it is possible that other granule components may account for yet other types of target cell damage.

Reactive Oxygen Intermediates

Roder and his colleagues have presented evidence that NK cells release reactive oxygen intermediates after target cell binding (163–165). The finding that NK activity (166, 167) and ADCC (168) are normal in patients with chronic granulomatous disease argues strongly against the hypothesis that reactive oxygen intermediates play a role in the LGL-mediated lethal hit. The results of Nathan et al (169), showed that this type of mechanism is

unlikely for CTL. Contaminating monocytes may have been responsible for the positive findings with NK cells (167). See note, p. 56.

Soluble Lytic Factors

Lymphotoxins are soluble factors found in the culture medium of lymphoid cell lines or of lymphocytes stimulated by antigens or mitogens. They have cytostatic and/or cytolytic effects on certain cells grown in vitro, particularly the fibroblast line L-929. The possibility that lymphotoxins are responsible for target cell damage in lytic processes of CTL and LGL requiring cell contact has long been debated, but some of the described properties of lymphotoxins have been difficult to reconcile with this role (170). The slow kinetics of lymphotoxin-induced target cell death, which requires a minimum of 5 hours to detect (171), stands in contrast to the rapid (within minutes) target cell death when it is mediated by cytotoxic lymphocytes (18, 172). Also, the specificity of α-lymphotoxin-induced cytolysis does not correspond to the antigenic specificity of the stimulus that gives rise to the lymphotoxin, so the lack of innocent bystander lysis is difficult to explain. Hiserodt et al (171) described an unstable, complex lymphotoxin that demonstrated the same allospecificity as its cell source, but their findings do not appear to have been confirmed subsequently. Several correlations between lymphotoxin action and cell-mediated cytotoxicity have been cited as evidence in favor of a role for lymphotoxin in such cytotoxicity (173), and it has been reported that antibodies against lymphotoxin preparations block CTL (50, 174) and NK (175) lytic functions. Other antilymphotoxin antibodies, however, fail to block CTL function (176, 177). The availability of cloned recombinant lymphotoxin should permit the production of defined antibodies and allow its biochemical definition.

Wright & Bonavida (178, 179) have described a lymphotoxin-like, soluble cytotoxic factor termed NK cytotoxic factor or NKCF, which is released into the medium by mouse and human lymphocytes after contact with tumor cells or mitogens. The hallmark of this factor is its target cell specificity, which correlates with that of the NK cells from which it is derived; particularly impressive in this regard is the correlation of cytotoxicity by NKCF and by NK cells on NK-resistant YAC tumor variants (180). While its NK-like specificity appears to distinguish NKCF from classical lymphotoxins, a direct comparison has not been reported. For both mouse and human systems, NKCF production requires LGL in the stimulated lymphocyte population (179, 181, 182), but similar cells have recently been reported to produce lymphotoxin (183). NKCF can be detected in lymphocyte lysates, but it requires a 4–48 hr culture period with

the stimulus for it to be detectable in the medium (184). While one laboratory has reported that NKCF production was only elicited by tumor cells infected by mycoplasma (185), Wright & Bonavida (186) have reported that NKCF production is elicited by a variety of stimuli, including mycoplasma-free cultured cells that are not NK targets.

Based on a series of correlations between NK cell–mediated lysis and NKCF lysis and between the conditions required to induce NKCF and the recognition event in NK killing, it has been proposed (187, 188) that NKCF is responsible for the lethal damage inflicted on target cells by NK cells. As is the case for lymphotoxins, the chief difficulty in accommodating NKCF into a model of NK-mediated cytolytic action is its slow kinetics. NKCF cytolytic activity can be detected after 16–20 hours of incubation with target cells, but target cell lysis increases until 40–70 hours (179, 182, 189). It has been argued that purer and more concentrated forms of NKCF may kill more rapidly or that a focusing mechanism operates to increase its local concentration (188). Another problem for the NKCF model of NK killing is the unclear relationship between NK-target binding and the postulated NKCF secretion, which can be triggered by many stimuli (186). Some of the correlations reported between NKCF and NK killing could be explained if NKCF were stored in LGL granules and exocytosed after target contact, but this would not mean that NKCF mediates the lethal hit inflicted by NK cells. Biochemical characterization of NKCF is necessary before its hypothesized role in NK cell killing and its possible relationships to lymphotoxin and LGL granule cytolysin can be tested rigorously.

Mediators Not Requiring Secretion

Largely because no plausible lytic mediators had been described, several proposals have emerged suggesting that target cell lysis occurs via physical forces or other mechanisms that would not involve a transfer of molecules from effector to target (2, 4, 5). In some EM studies, breaks in the target membrane have been seen after incubation of CTL-target pairs (190), leading to suggestions of membrane damage by shear forces. Critical experiments addressing these mechanisms are hard to design and have not been carried out. Careful experiments did not reveal any transfer of molecules by cytoplasmic junctions between effector and target cells (160).

Some investigators have considered the possibility that the plasma membrane of cytotoxic lymphocytes is the source of the lethal damage inflicted on target cells (191, 192). Recent experiments in which reconstituted material from CTL transferred cytolytic capacity to noncytologic cells by membrane fusion were interpreted as support for this concept (193). However, these experiments need to be repeated using more

defined reconstitution systems and recipient cells that clearly do not have cytolysin-containing granules before we can conclude that CTL plasma membranes are responsible for their lytic activity.

CONCLUSIONS

At our current state of knowledge, it is clearly premature to conclude that any one model can explain all known aspects of lymphocyte-cytotoxic mechanisms. As outlined above, many of the more recent studies have tended to support the hypothesis that a secretory process, and in particular the granule exocytosis model, explains both CTL- and LGL-mediated cytotoxicity. This model is illustrated in Figure 1 and includes the cytoplasmic rearrangement and granule exocytosis steps described above. It is similar to models presented previously (1, 97) but has taken on new significance with the demonstration of the lytic properties of the granules. The potency and other properties of the membrane-pore-forming granule cytolysins make them the only plausible, experimentally demonstrable lytic mediators from cytotoxic lymphocytes. However, the granule exocytosis model allows for other types of target cell damage, which can be inflicted by

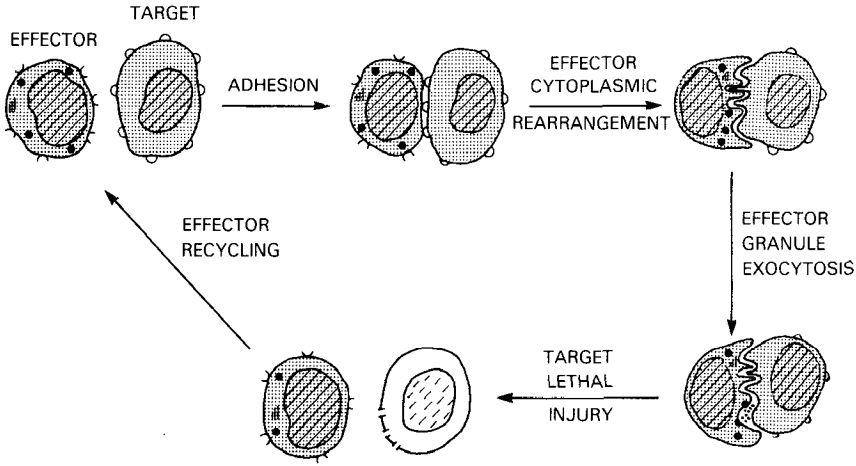

Figure 1 Granule exocytosis model of lymphocyte-mediated cytotoxicity. This model shows the adhesion (i.e. conjugate formation) phase of the process described previously. This is followed by the effector-cell cytoplasmic rearrangement and granule exocytosis steps. The latter would clearly be part of the programming for lysis stage, while cytoplasmic rearrangement could be part of the adhesion or, more likely, the programming stage. The final stage, KCIL, results in a lethal injury to the target cell, while the effector cell can recycle and kill another target.

other granule components after the pores are implanted in the target membrane. As outlined above, the granule exocytosis model is supported by many different lines of evidence, and the arguments against this type of mechanism do not appear to be strong. There are several issues that must be addressed experimentally before for the granule exocytosis model can be accepted completely. Among these are the kinetics of target cell death induced by the granule cytolysins vs by cytotoxic lymphocytes and the question of why the killer cells do not die after granule exocytosis. Critical experiments that explore these questions and characterize cytotoxic lymphocyte granules in more detail are underway in several laboratories.

The concept of a secretory mechanism, such as the granule exocytosis model, for lymphocyte-mediated cytotoxicity is satisfying in a broader context because it then becomes possible to regard lymphocyte effector mechanisms in general as being secretory. The secretion of immunoglobulin by plasma cells is clearly the end result of the differentiation of B lymphocytes. In a similar way, one can view the differentiation of T lymphocytes as a specialization to secrete lymphokines or other biologically active factors. While some of these may have an antigenic specificity, others may be molecularly nonspecific but act locally. For functions such as cytotoxicity where rapid secretion is advantageous, such mediators may be stored intracellularly in granules. In other cases, such as antibody secretion by plasma cells, there may be no significant storage. The current rapid pace of molecular biology and the availability of clones and hybridomas makes the prospect of defining such secretory products an exciting one for future study.

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NOTE ADDED IN PROOF Pohajdak et al (194) have recently shown that NK-sensitive target cells rapidly induce purified LGL to release a soluble factor that triggers the respiratory burst in monocytes. These results may provide an explanation for the chemiluminescence reported to be associated with NK cytolysis (163–165) if monocyte depletion was incomplete in the original experiments.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 59–85

HYPERSENSITIVITY LUNG DISEASE

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INTRODUCTION

The immunology of the lung in both health and disease has been a major focus of research in recent years. As a result, many pulmonary syndromes previously defined by clinical and pathological descriptions can now be categorized by immunologic processes that appear to contribute to the abnormal state. In this review I examine recent studies of hypersensitivity lung disease, concentrating on observations involving both human disease and animal models of disease. I emphasize the evolution of our understanding of basic immunologic reactions within the lung.

Rather than list all pulmonary disorders where immunologic mechanisms may contribute to the process, I stress general concepts and discuss the more common lung diseases. It is apparent that the disease processes are complex, that our knowledge in many areas is incomplete, and that no simple method of categorizing lung diseases with an immunologic basis is possible.

The lack of a satisfactory system of classification of hypersensitivity reactions deserves early comment. The classification of allergic reactions introduced by Coombs & Gell is now over 20 years old (1). While the actual reactions are more complex than the four types initially described, and although the categories tend to oversimplify pathologic states when applied to human disease, the Coombs & Gell scheme does offer a basic framework for discussion and is therefore employed in this review. In addition, examination of how our thinking about these reactions has changed over two decades helps us appreciate the evolution of knowledge in the fields of immunology and lung disease.

Some general comments about the lung should be made. The lung is a

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complex organ that comes in contact with large volumes of air to accomplish its primary task of gas exchange. The airways (trachea, bronchi, bronchioles) that lead to the gas exchanging units (alveoli) as well as the alveolar structures themselves are continually exposed to potentially pathogenic material in our environment. Thus, various antigens and infectious or noxious agents commonly present themselves to the lung where they can interact with pulmonary cells and proteins that are capable of producing an immune or inflammatory response. In addition, through its extensive vasculature the lung is exposed to virtually all the circulating cells and proteins that can mount an inflammatory reaction in response to an immunologic stimulus. In general, these reactions protect the host from infection and eliminate foreign material (2). However, for reasons that are poorly understood, these same humoral and cellular events can also lead to damage. When this occurs, the lung is vulnerable to attack on two fronts: through the airways and air-spaces, or through the pulmonary vasculature. Both are potential routes of inflicting damage, as will be seen with the human and animal diseases to be discussed.

CLASSIFICATION OF ALLERGIC REACTIONS

The Coombs & Gell classification of immunologic responses has been used by various authors to define the immune reactions responsible for pulmonary hypersensitivity diseases (3–5). The four immunologic responses are: type I reactions that are IgE dependent, type II cytolytic or cytotoxic reactions, type III or Arthus-type reactions (immune complex disease), and type IV reactions that refer to delayed or "tuberculin-type" hypersensitivity. That these processes are more complex than initially described can be appreciated by comparing a later edition of *Clinical Aspects of Immunology* (6) to the original (1). While researchers can more or less isolate these immunologic reactions in pure form in some animal models, the situation in humans is much more complex. Indeed, in their original description of the four reactions, Coombs & Gell noted "it is an easy error to assume that, even when one process is demonstrable, this process, and only this, is responsible for the whole trouble, or indeed any of it" (1). These authors realized that in allergic disease as well as other diseases, a number of different processes may be occurring simultaneously. Thus, while it is possible for one type of immunologic reaction to dominate, it is an oversimplification to believe that a hypersensitivity lung disease is a manifestation of a single immune mechanism. Furthermore nonimmune mechanisms may contribute to many of the disease states to be discussed.

In the following paragraphs, I briefly review the four types of allergic reactions, citing studies on these immunologic responses in various organs or tissues. I then note examples of human lung disease involving these reactions.

Type I Reactions

Our understanding of the pathogenesis of type I reactions has changed dramatically since the original classification of these anaphylactic or reagin-dependent responses. This is in part due to the description of a class of immunoglobulin termed IgE (7). While some evidence has been presented that IgG_4 antibody can sensitize mast cells and lead to hypersensitivity disease (8), it is apparent that the primary carrier of reaginic activity in man is the IgE.

The type I reaction has been termed immediate hypersensitivity and involves the release of mediators from IgE-sensitized mast cells or basophils, leading to increased vascular permeability, edema, and smooth-muscle contraction (6, 9, 10). Serum from an allergic patient can sensitize skin from a normal individual to a specific antigen in vivo (Prausnitz-Kustner test) as well as lung fragments from a nonallergic donor in vitro (11). This sensitizing activity is mediated by antibodies of the IgE class and not other antibody classes, as shown by Ishizaka & Ishizaka (12) with biochemical techniques and absorptions with specific antisera. IgE exerts its effect by attaching to specific receptors on basophils and mast cells, with initiation of reactions occurring when the antigen bridges two or more cell-bound IgE molecules (6).

IgE–DEPENDENT LATE–PHASE RESPONSES A major reorientation in thinking about IgE-mediated hypersensitivity has occurred in recent years. Researchers realized that this class of immunoglobulin can mediate not only immediate but also delayed events. This phenomenon was first studied in detail in skin, and later was evaluated in the lung. I review these observations in turn.

The observation that allergic patients exposed to allergens can develop both early (immediate) and late responses in skin dates back six decades. In passive transfer experiments, Prausnitz & Kustner (13) noted that the cutaneous response to antigen challenge could last several hours. The pathogenesis of the late cutaneous response (LCR) was investigated by Pepys and co-workers (14), who noted that the late skin response to *Aspergillus* in patients with allergic bronchopulmonary aspergillosis showed deposition of IgG, IgM, and C3.[1] This led to the conclusion that an

[1] Abbreviations: ABM, alveolar basement membrane; C_{dyn}, dynamic compliance; C3, the third component of complement; C5, the fifth component of complement; C5a, the C5 fragment with anaphylatoxin activity; C5a des Arg, the C5 fragment formed by removal of the C-terminal arginine from C5a; C5+, C5 sufficient B10.D2/nSn mice; C5−, C5 deficient B10.D2/oSn mice; GBM, glomerular basement membrane; IAR, immediate asthmatic response; ICR, immediate cutaneous response; IPF, idiopathic pulmonary fibrosis; LAR, late asthmatic response; LCR, Late cutaneous response; PCA, passive cutaneous anaphylaxis; R_L, pulmonary resistance.

Arthus reaction (type III) was responsible for the continuing inflammation. However, several recent observations regarding LCRs have suggested that many of these reactions are not type III events. For example, Dolovich & Little (15) showed that LCRs occurring after injection of *Bacillus subtilis* enzymes did not appear to require antibodies other than IgE. Dolovich and co-workers (16) showed that LCRs also occurred after injection of anti-IgE, demonstrating that complement activation was not essential to the response. The hypothesis that LCRs could be mediated by IgE-related mechanisms was further strengthened by Solley and co-workers (17) in experiments where heating of the passively transferred serum for 4 hr at 56°C to inactivate IgE led to ablation of the capacity to transfer both the immediate cutaneous response (ICR) and the LCR. Removal of IgE by passage of serum over an anti-IgE immunoadsorbent also abolished the ability to transfer the reactions passively. When passively transferred, the IgE recovered from the immunoadsorbent restored the responses. These workers also observed that serum from ragweed-sensitive subjects, when mixed with large amounts of myeloma IgE, led to blockage of the ICR and LCR. Zetterstrom (18) subsequently reported that passive transfer of antigen-specific IgG antibody followed by challenge with specific antigen did not lead to LCRs while transfer of antigen-specific IgE did. He also observed that mixtures of IgE and IgG gave smaller LCRs in three of four tests, raising the possibility that IgG might actually suppress rather than enhance the reactions.

The studies showing the dependence of the LCR on IgE suggested a role for mast cells and basophils in these reactions. Both Solley and co-workers (17) and de Shazo and associates (19) found that injection of Compound 48/80, a substance known to trigger mast-cell degranulation (20), produced lesions similar to LCRs. Further support for the central role of the mast cell in producing LCRs came from Kaliner and co-workers (21, 22), who found that intracutaneous injection of rat peritoneal mast-cell granules into rats caused the same lesion as injection of anti-IgE or Compound 48/80. From an ultrafiltrate of mast-cell granules, these investigators recently isolated an undecapeptide that may help produce the early polymorphonuclear neutrophil influx that is followed by a later mononuclear cell infiltration characteristic of late-phase allergic responses in rat skin. Thus, the experiments on the LCR during the last decade strongly suggest that IgE and the mast cell are central elements in the production of this response.

Results on the histopathology of this IgE-dependent late response to antigen in the skin in one experimental animal, the rat were described above. Behrens and co-workers (23) have recently described the pathology of an immediate and late cutaneous response in rabbits challenged with *Alternaria tenuis*, an antigen to which the animals had only or predomi-

nantly antigen-specific IgE present in their serum. Interstitial edema and vessel dilatation were present 30 min after intradermal challenge, while at 6 hr a perivascular cellular infiltrate was noted. By 48 hr, a mixed cellular infiltrate (mononuclear and polymorphonuclear cells) was seen. In clinical studies, Dolovich and co-workers (16) found that LCRs induced with either ragweed antigen or anti-IgE were characterized by edema, mast-cell degranulation, and cellular infiltration with eosinophils and neutrophils 6 hr after challenge. Immunofluorescent studies failed to reveal deposition of IgG, IgM, IgA, or B_1C. Solley and associates (17) also found that the LCR was characterized by edema and a mixed cellular infiltrate, and not deposition of immunoglobulins or complement. Thus, both in experimental animals (rats and rabbits) and in clinical studies, the late cutaneous response, a response associated with IgE-antigen interaction, is characterized by an inflammatory response in the target organ (skin).

ASTHMA Asthma is a common chronic lung disease characterized by intermittent, reversible obstruction of the airways. In patients with asthma who develop symptoms after allergen exposure, different temporal patterns of obstruction are recognized. These include an immediate asthmatic response (IAR), which occurs 10–30 min after allergen challenge and represents an IgE-mediated reaction to allergen, and a late asthmatic response (LAR), which occurs 2–12 hr after allergen exposure. In reviews of hypersensitivity lung disease, the classic example of pulmonary immediate hypersensitivity has been the immediate asthmatic response induced by antigen (3–5). While the initiation (IgE plus antigen) of this type of immediate response (type I) is not in question, the relative importance of an IAR in the more severe forms of asthma is arguable. Several observations suggest that this late asthmatic response should be thoroughly studied in order to understand the patient with asthma. For example, LARs resemble chronic, severe bronchial asthma in that steroids are required to decrease or abolish symptoms and abnormalities in pulmonary physiology (24, 25). In addition, Warner (26) has observed a significant correlation between frequent attacks of asthma and the presence of LARs in children challenged with house dust mite. Warner and co-workers (27) also noted that clinical improvement after immunotherapy with extracts of *Dermatophagoides pteronyssinus* was associated in many patients with loss of the LAR but not the IAR on rechallenge. Furthermore, Cockcroft and associates (28) have reported that allergen-induced LARs are associated with an increase in bronchial responsiveness to histamine and methacholine while IARs do not correlate with this heightened responsiveness. Cartier and associates (29) reconfirmed the association between LARs and an increase in bronchial responsiveness to histamine and demonstrated that the increased re-

sponsiveness is not due to a reduction in baseline airway caliber alone. Boulet and co-workers (30) have also shown that the ragweed-sensitive asthmatics with LARs and not just IARs have the greatest increases in airways reactivity after natural exposure to ragweed. Thus, LARs not only clinically resemble chronic asthma, they also appear to be related to a basic feature of asthma: heightened airway reactivity.

Because the LAR occurs at the same time as the LCR, and because until recently the LCR was thought to be an Arthus-like reaction, it was assumed that the LAR was a type III response (31). However, several clinical observations suggest that LARs, like LCRs, are not all Arthus reactions. First, pollen antigens such as ragweed, which ordinarily do not cause production of large quantities of IgG antibody, can provoke LARs (32). Second, inhalation of cromolyn sodium prior to antigen inhalation will prevent both IARs and LARs, thus implicating the mast cell in the pathogenesis of LARs (24). Third, high-molecular-weight neutrophil chemotactic activity, thought to be mast-cell associated (33), was demonstrated by Atkins and co-workers (34, 35) in the serum of ragweed-sensitive subjects shortly after bronchial provocation. This activity has recently been demonstrated by Nagy and co-workers (36) to appear in two peaks in the sera of patients undergoing allergen bronchial provocation. These peaks coincide with the early and late allergen-induced pulmonary reactions, again suggesting that mast cells may participate in both responses. Fourth, the effect of steroids on LARs parallels that seen with LCRs: the immediate reaction is not altered while the late response is abolished (37), suggesting that the skin and lung may have more in common mechanistically than just the temporal development of the reaction. Thus, it is logical to speculate that late asthmatic responses, like late cutaneous responses, might be dependent on IgE and not Arthus reactions.

Testing this hypothesis is not possible in man, but from the work of Zvaifler & Robinson (38) as well as Ishizaka and co-workers (39) it is known that rabbits produce a homocytotropic antibody analogous to human IgE. Pinckard and associates (40) reported that many rabbits immunized with antigen within the first 24 hr of life will produce IgE (homocytotropic antibody) to that antigen exclusively whereas rabbits immunized first at 7 days of age will produce several antibody isotypes to the antigen. Thus, Shampain and associates (41) immunized rabbits from birth with *Alternaria tenuis*, a mold known to cause LARs in humans. At 3 months of age, we had a group of rabbits with only antigen-specific IgE to *Alternaria* as defined by homologous passive cutaneous anaphylaxis (PCA) performed after a latent period of 3 days (42). Another group of rabbits had not only antigen-specific IgE to *Alternaria* but also anti-*Alternaria* IgG as defined both by heterologous PCA in guinea pigs and by precipitin assay (43). Upon aerosol

challenge of these groups of animals with *Alternaria* antigen, the group with only IgE antibody to the antigen showed immediate and late alterations in pulmonary resistance and dynamic compliance consistent with increased airways obstruction (Figure 1). Nonimmunized rabbits challenged with either saline or *Alternaria tenuis*, or rabbits immunized with bovine serum albumin and challenged with saline or *Alternaria*, did not exhibit significant

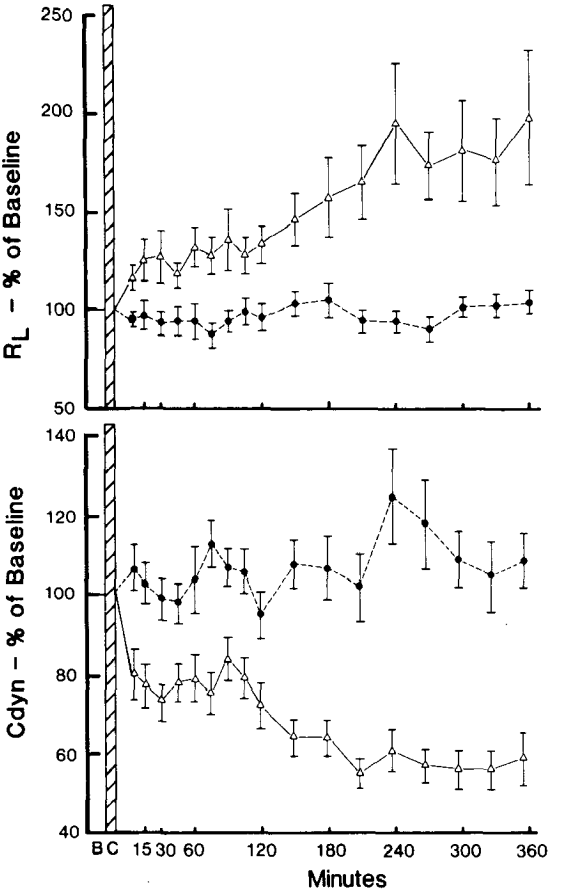

Figure 1 Changes in pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) expressed as a percentage of baseline (B) after challenge (C) with *Alternaria tenuis* aerosol. The 19 rabbits with anti-*Alternaria* IgE antibody without antigen-specific IgG are shown in open triangles while the 10 controls (nonimmunized or immunized with bovine serum albumin) are shown by the filled circles. Means ± SEM are shown. In the rabbits with anti-*Alternaria* IgE, significant changes in lung function consistent with increases in airways obstruction were seen a few minutes after challenge and increased in severity with time. No significant changes in lung function occurred in controls at any time. Reprinted with permission from Shampain et al (41).

alterations in lung function (41). Thus, in rabbits, late obstruction of airways after antigen challenge occurred when only antigen-specific IgE was present and did not appear to depend on the presence of IgG, again arguing that late airways obstruction need not be an Arthus reaction.

In the same study, rabbits with both antigen-specific IgE and IgG had early and late responses to antigen challenge, but these responses were weaker than those seen in rabbits with IgE alone. To determine if the presence of antigen-specific IgG might be inhibiting the LAR, rabbits with only IgE were directly compared to rabbits with both higher titers of IgE and detectable IgG. Despite having lower IgE titers, animals with only IgE had stronger responses than rabbits with IgE and IgG. Passive transfusion of serum containing IgG against *Alternaria* into rabbits with only antigen-specific IgE blunted the response of the animals to challenge, again demonstrating that IgG inhibited the reaction. Similar observations have been made in rabbits sensitized with ragweed (44), showing that this phenomenon is not restricted to one antigen.

Studies have also been conducted in animals passively sensitized by transfusion of serum containing antigen-specific IgE and/or IgG to determine if similar events occur in the absence of cellular immune mechanisms associated with immunization (41, 44). Passively sensitized rabbits receiving only antigen-specific IgE had LARs; and in the presence of both IgE and IgG, the LAR was diminished. In another study, Behrens and co-workers noted that as the titer of transfused antigen-specific IgG increased, the magnitude of the LAR decreased despite higher IgE titers (45). In addition, transfusion of rabbit serum with both antigen-specific IgE and IgG that was heat treated to minimize IgE led to no pulmonary response on antigen challenge. Fluorescent antibodies to complement and immunoglobulin failed to show evidence of a type III response in any group of animals. Thus, in both actively and passively sensitized animals, the presence of antigen-specific IgE led to LARs while the presence of antigen-specific IgG blunted the response.

The histopathology of the lungs in rabbits after antigen challenge parallels findings in the skin. The IAR is characterized by submucosal edema and vessel dilatation while the LAR is characterized by cellular infiltration of the airways down to and including the bronchioles (23). The histology of human airways during a developing IAR and LAR is not known; our knowledge is limited to histologic descriptions of the lung from biopsy or autopsy specimens from patients with asthma (46). Given the similarity of cutaneous findings after antigen challenge in rabbit and man, future studies in rabbits may offer insight into the pathology of the process in man.

Investigation of the LAR in clinical studies as cited above and through

use of animal models in our laboratory (23, 41, 44, 45), as well as in other laboratories (47), is directed towards understanding the mechanism of late, antigen-induced, airways obstruction, a response that closely resembles chronic, severe asthma. It can be argued that the disease of many steroid-dependent asthmatics has no definable "allergic" trigger and thus mechanisms that cause LARs after antigen challenge may not apply to them. However, other observations point toward a possible link, in terms of the pathogenesis of airways obstruction, between patients with and without allergic triggers. For example, asthma precipitated by exercise has been associated with rises in both circulating neutrophil chemotactic activity (48) and histamine (49) in atopic asthmatics. This suggests that nonallergic triggers such as exercise may lead to release of mast-cell mediators. In addition, Lee and associates (50) have noted elevations of plasma histamine and of high-molecular-weight neutrophil chemotactic activity in sera of both atopic and nonatopic asthmatics with exercise-induced asthma. It is thus possible that release of mast cell–associated mediators may be independent of the atopic state. Therefore, future information obtained by IgE-mediated release of mediators may apply to mediator release induced by other stimuli.

The studies reviewed above of late responses in skin and lung indicate that they can be IgE dependent and not Arthus reactions. The studies also point out that IgE mediates responses that are not just "immediate" in timing. However, tissues responses that occur at approximately the same time after exposure to antigens or noxious agents can reflect other immunopathologic events, such as an Arthus reaction (see below).

Type II Reactions

The Coombs & Gell type II or cytotoxic antibody-mediated reaction involves IgG or IgM antibody directed against a cell surface or an antigen attached to a cell. The mechanism by which specific antibody produces tissue damage includes the union of antibody with antigen, leading to complement activation. This in turn attracts inflammatory cells such as polymorphonuclear leukocytes to the region, where they help produce tissue injury (5). The diagnosis of this type of pathology is based on demonstrating tissue-specific antibody either in the circulation or at the site of injury. Recently, Henson (51) suggested that type II reactions, where antibody reacts with host tissue–associated antigens, and type III reactions, where antibody reacts with free antigens of either exogenous or host origin, should be considered together under the heading of antibody-mediated inflammation. This recommendation was based in part on the fact that these processes mediate tissue injury by similar mechanisms. In line with this concept, the mechanisms through which injury is produced are

considered in more detail below, in the section on type III responses. On the other hand, because Goodpasture's syndrome has been cited in past reviews as the classic example of a type II reaction, and because new clinical and experimental data on this syndrome illustrate important points regarding hypersensitivity reactions in the lung, this clinical problem is now discussed separately under its older designation as an example of a type II response.

GOODPASTURE'S SYNDROME Stanton & Tange (52) initially suggested the term "Goodpasture's syndrome" in 1958 for the clinical syndrome of intra-alveolar hemorrhage and necrotizing glomerulonephritis similar to that described by E. W. Goodpasture in 1919 (53). Rees (54) indicates that the pathogenesis of this syndrome was unknown until autoantibodies to basement membranes were demonstrated with linear deposits of IgG on glomerular and alveolar basement membranes. Thus far, the component of basement membrane that antibody binds to has not been formally identified (55). Collagenase digests of both glomerular and alveolar basement membranes have been employed to study the biochemical nature of these antigenic determinants, and it appears these basement membrane autoantibodies are directed against glycoproteins with a single or possibly a few antigenic determinant(s) (54).

The pathogenicity of circulating antibodies to glomerular basement membrane (GBM) has been demonstrated in many ways. For example, in passive transfer experiments, Lerner and co-workers (56) demonstrated that anti-GBM antibodies from humans could transfer acute glomerulonephritis to normal recipient monkeys. In addition, patients with high anti-GBM antibodies developed immediate recurrence of glomerulonephritis after kidney transplantation (57). In the lung, this pathogenicity has been harder to prove. Intravenous injection of antibodies against alveolar basement membrane (ABM) in various species has not consistently led to binding within the lung (54, 58, 59). This appears to be due to a relative impermeability of the alveolar capillaries to molecules the size of IgG under normal conditions (60). Binding of IgG to the ABM is usually demonstrated in patients with Goodpasture's syndrome (61); this has led to the proposal that alveolar capillary permeability must increase before circulating antibodies can gain access to the ABM. Both clinically (62) and experimentally (59), we know that certain factors increase alveolar capillary endothelial permeability. Hyperoxia, bacteremia, endotoxemia, and increased capillary hydrostatic pressure (63)—all increase the pulmonary problems associated with Goodpasture's syndrome. In addition, Donaghy & Rees (64) recently reported a strong association of smoking and pulmonary hemorrhage in patients with autoantibodies to GBM, which may be related to alterations in capillary permeability due to smoking.

Thus, circulating antibody that will bind to ABM will not by itself result in pulmonary disease; another stimulus may be needed.

Unfortunately, there are no data to identify the cause of synthesis of these antibodies against basement membrane. Researchers have noted an association of Goodpasture's syndrome with unusual exposure to volatile hydrocarbons (65), and speculated that the first stage in the pathogenesis of the disorder is chemical injury to lung or kidney, followed by production of antibodies to altered basement membrane in one of the organs. However, a history of such exposure is uncommon. On the other hand, approximately one half of patients with this disease have a history of an upper respiratory tract infection immediately before the onset of symptoms, although an association with a well-defined respiratory pathogen such as influenza A_2 is unusual (66). Thus, these associations appear real; but it is presently impossible to distinguish between (*a*) direct effects on ABM or GBM that may initiate antibody synthesis and (*b*) secondary effects, such as alteration of capillary permeability or amplification of an inflammatory process, that lead to disease expression.

Regardless of what initiates antibody synthesis, there is now strong evidence of a genetic contribution to disease susceptibility. Nearly 90% of patients with Goodpasture's syndrome in the United Kingdom have the major histocompatability antigen HLA-DR2 (67), which appears to determine susceptibility. In addition, patients who have HLA-B7 together with DR2 have the disease more severely (68). In the Brown-Norway rat, an animal model of mercuric chloride–induced nephritis associated with anti-GBM antibodies, genetic control of the immune response is also important (69). These autoimmune abnormalities are transient (70), suggesting that spontaneous suppressive mechanisms normally develop. In fact, spleen cells from rats that have recovered from the disease prevent or delay the disease when transferred to normal syngeneic recipients; this suggests suppressor cells may be important in preventing the development of this pathologic process (71). Further study of the induction and regulation of normal and pathologic immune responses in man, and models such as this, will contribute to our understanding of this disease.

Type III Reactions

As reviewed above, type II and III reactions of the old Coombs & Gell classification have recently been grouped together under the heading of antibody-mediated inflammation (51). Pathologically these two classes can be distinguished by the fact that immune complex disease (type III) is more likely to produce unevenly distributed focal or nodular deposits of electron-dense material containing antigen, antibody, and complement along the surface of the target tissue, whereas type II injury is characterized by an

even, linear deposition of antibody and complement along tissue surfaces. Nevertheless, the pathogenic mechanisms leading to injury are similar. Thus, in terms of Goodpasture's syndrome, binding of anti-GBM antibody causes little or no damage unless inflammatory mediators and cells such as complement and neutrophils bind to exposed Fc regions after a critical mass of antibody has been fixed (54, 72). Indeed, there is little evidence that in the absence of Fc-dependent effector systems immune complexes promote injury. For example, Scherzer & Ward (73), as well as Roska and co-workers (74), studied the importance of the complement system in immune complex lung disease by depleting complement with cobra venom factor. The former investigators found permeability and hemorrhage decreased toward control values when the treated rats were challenged with preformed immune complexes. Roska and associates found complement depletion of ovalbumin-sensitized guinea pigs led to suppressed inflammatory reactions after aerosol challenge with specific antigen. We have also reported that immune complexes instilled into the lungs of mice produce significantly more pulmonary inflammation in a C5-sufficient strain (B10.D2/nSn) than in the congenic C5-deficient strain (B10.D2/oSn), as defined by lavage (Figure 2) and histology (75). Thus, the complement system in general, and C5-fragments such as C5a and C5a des Arg in particular (76), appear to be an important component of the inflammatory response induced by antigen-antibody complexes.

Animal models of immune complex lung disease have shown that the inflammatory responses depend not only on complement as outlined above, but also on neutrophils (77). The neutrophils are capable of

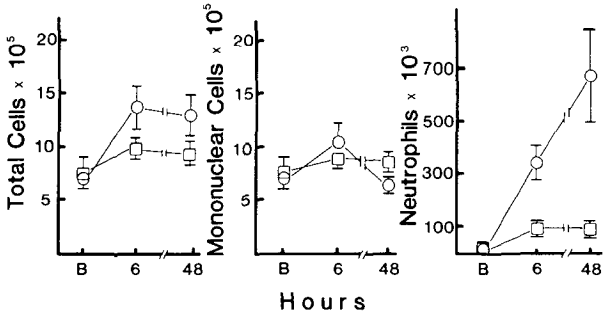

Figure 2 Cells in pulmonary lavage before (B = baseline) and 6 and 48 hr after intrapulmonary challenge with immune complexes in C5+ (circle) and C5− (square) mice. The points are means ± SEM. Significant increases in total cells occurred after challenge in C5+ mice, with the changes due to greater increases in lavage neutrophils in the C5+ mice at both times. The number of mice examined at any one time was 5–9. Reproduced with permission from Larsen et al (75).

producing tissue injury through multiple mechanisms. Two of these currently receiving much attention, are the release of proteolytic enzymes and the production of toxic oxygen metabolites. In terms of the former mechanism of tissue injury, Johnson and co-workers (78) found that, in normal mice and in "beige" mice with deficient neutral protease activity in their neutrophils, dermal vasculitis induced by immune complexes was comparable in terms of injury when assessed by histology and vasopermeability. In the lung, Johnson & Ward (79) found that intratracheal installation of protease inhibitors (soybean trypsin inhibitor, alpha$_1$ antitrypsin inhibitor from human serum) into rats did not protect against lung injury induced by immune complex while airway instillation of catalase did provide protection. As reviewed by Fantone & Ward (80), observations such as these have led to interest in the potentially injurious role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions in many tissues. In experimental immune-complex lung injury in the rat, superoxide dismutase has a brief (2-hr) suppressive effect on the response that is associated with a lack of influx of neutrophils (81); catalase more effectively prevents injury while not blocking the influx of neutrophils (79). These observations suggest that production of the superoxide anion (O_2^-) by leukocytes leads to generation of an oxidation product of arachidonic acid with chemotactic properties (82), while hydrogen peroxide (H_2O_2) is more directly responsible for tissue injury. The cell most often implicated as of pathogenic importance in immune complex lung disease has been the neutrophil, but rat alveolar macrophages as well as neutrophils produce significant amounts of O_2^- and H_2O_2 upon exposure to immune complexes in vitro as well as in vivo (83). The relative importance of each cell type, especially the alveolar macrophage, to tissue injury associated with immune complexes remains to be further delineated.

The potential role of immune complexes in human diseases in general (84) and human lung disease in particular (85) has been recently reviewed, with particular attention to the demonstration of immune complexes in various tissues and biologic fluids in the body. While the presence of such complexes has been used to characterize various disorders, the pathogenetic significance of the complexes is often uncertain, a point discussed below. It must be stated from the beginning that the formation of immune complexes is usually beneficial to the host, and results in the neutralization and/or elimination of an antigen. Thus, the demonstration of these complexes is not synonymous with pathology. These complexes are usually managed by the reticuloendothelial system, but under various circumstances, they may not be eliminated, or may be deposited or formed in vascular structures where a prolonged inflammatory process may be

established. The factors that determine whether immune complexes are protective or cause and/or contribute to disease are not well defined, but certainly they include the nature of the antibody and antigen, the relative concentrations of each that lead to formation of complexes, the state of the phagocytic system, the location of the complexes in the body, and genetic factors in the host.

A review of the various methods employed to detect immune complexes in the circulation or tissues of the body is beyond the scope of this review. Recent publications dealing with immune complex disease address this subject in detail (84, 85). Suffice it to say that the various methods available for detecting immune complexes differ in sensitivity as well as in the information they provide, making it difficult at times to compare results from different laboratories. The possible advantages as well as the limitations of these various assays must be kept in mind in interpreting the results of studies.

Immune complex deposition in the respiratory tract may take place via one of several routes. The complexes could form systemically at a point far from the lung and be deposited in the lung via the blood stream. In animal models, merely injecting preformed complexes intravenously does not lead to tissue deposition unless maneuvers leading to increases in vascular permeability are also employed (85, 86). Alternatively, the subject may inhale antigens which encounter specific antibody produced locally in the lung by lymphocytes either in the bronchus-associated lymphoid tissue (87) or in the lower respiratory tract, as demonstrated in bronchoalveolar lavage fluids (88). In addition, lung antigen that has been altered may be recognized as foreign and lead to antibody production locally and/or systemically. Finally, antigen could be carried to the lung via the circulation, there encountering locally produced antigen-specific antibody. The fact that the antigens involved in the production of immune complexes are unknown for many diseases means we can only guess about which mechanism led to formation of the complexes.

As noted above, the pulmonary diseases associated with the demonstration of immune complexes is heterogeneous and growing larger. For example, immune complexes have been demonstrated in cystic fibrosis (89, 90), bronchogenic carcinoma (91), mycobacterial infections (92), invasive fungal diseases (93), and sarcoidosis (94) as well as many collagen-vascular diseases (85). Because of space limitations, I review here only two general groups of diseases where immune complexes have been demonstrated: idiopathic pulmonary fibrosis and hypersensitivity pneumonitis.

IDIOPATHIC PULMONARY FIBROSIS One of a group of interstitial lung diseases of unknown etiology (95), idiopathic pulmonary fibrosis usually

presents clinically with dyspnea and cough in patients in their fifth or sixth decade of life, although children and the elderly can also be affected. The course of the disease is progressive and usually fatal within an average of 3–6 yr from the onset of symptoms (95, 96). Chest X rays typically show reticulonodular infiltrates while assessment of lung function shows a restrictive pattern. After excluding known causes of interstitial lung disease (97), open lung biopsy is the procedure of choice in establishing the diagnosis (98). The histology varies with the stage of the disease; an early alveolitis characterized by accumulation of inflammatory and immune effector cells is followed by thickening and fibrosis of alveolar walls with an increase in interstitial collagen. In addition to establishing a diagnosis, the biopsy can offer prognostic information. Specimens displaying greater cellularity augur better responses to therapy while a fibrotic picture leads to a more guarded prognosis.

There are many observations that suggest immune complexes may be important in the pathogenesis of this disease. First, immunofluorescence studies have shown that immunoglobulin and complement are deposited in the alveolar wall (99). Second, immune complexes have been found in the blood and lavage fluid of patients with marked alveolitis (99–101). In the study by Dreisin and associates (99), levels of circulating immune complexes as detected by the Raji cell radioimmunoassay (102) were usually elevated in patients with cellular stages of the disease but were not detectable when diffuse fibrosis was present. In addition, significant intrapulmonary deposition of IgG and C3 was limited to patients with circulating complexes, suggesting these complexes might be related to pathogenesis. This notion was strengthened by the observation that steroid therapy usually improved the clinical course in patients who initially had levels of circulating complexes, and these levels fell with steroid treatment (85, 99). Third, alveolar macrophages obtained from patients with IPF have IgG Fc and C3b receptors occupied, which is consistent with the presence of immune complexes on their surface (101, 103).

Although the antigen(s) in the immune complexes has not been identified, components of the alveolar wall such as type I collagen have been proposed as candidates (95, 97). Hunninghake and associates (104) have reported that approximately one third of these patients have lung lymphocytes that spontaneously secrete immunoglobulin specific for type I collagen. Morphologic studies have shown that type I collagen of alveolar-walls is deranged as well as increased in amount in patients with IPF (105). The observation that blood lymphocytes from these patients release lymphokines on exposure to type I collagen (106) also raises questions about the role of collagen in producing disease.

Crystal and co-workers have proposed that the alveolar macrophage is a

central cell in directing the alveolitis (95). Thus, this cell secretes one or more chemotactic factors for neutrophils (107, 108); releases mediators that expand the population of fibroblasts (109, 110); and produces increased amounts of fibronectin (111), a chemoattractant for fibroblasts (112). Although the alveolar macrophage may help orchestrate many of these events, the neutrophil is thought to cause much of the damage to the cells in the alveolar wall (95). While the above outline provides an attractive and plausible scenario, it must be emphasized that central questions remain. For example, is there a common unrecognized precipitating event that leads to this disease process? Is the presence of immune complexes an epiphenomenon or a primary mechanism of producing tissue injury? What role does complement play in the human disease? Animal experiments reviewed above suggest immune complex–induced injury is very dependent on complement, but current popular theories based on clinical studies suggest it may not be critical.

HYPERSENSITIVITY PNEUMONITIS In contrast to idiopathic pulmonary fibrosis where the inciting agent(s) that causes the interstitial disease is not defined, the antigens that can cause hypersensitivity pneumonitis (extrinsic allergic alveolitis) are better delineated. In recent years, a number of antigens responsible for the syndrome have been identified, including various microorganisms, plant and animal proteins, and low-molecular-weight chemicals and drugs (113). Researchers recognize acute, subacute, and chronic forms of the disease, which are reflections both of the agents involved and of the intensity and duration of exposure. In the acute form of the disease, 4–12 hr after exposure, the individual develops constitutional symptoms (increased temperature, myalgias and arthralgias) as well as symptoms referrable to the respiratory tract (cough and dyspnea, but not usually wheezing). Rales are evident on physical examination of the lungs; the chest X ray ranges from normal to demonstrated nodular infiltrates in the lower lung fields (113). Pulmonary function tests generally show a restrictive pattern (4). Subacute or chronic forms of the disease are associated with symptoms of dyspnea, cough, and weight loss with X rays showing evidence of interstitial fibrosis. While lung function is most commonly restrictive in pattern, an obstructive element with decreased flows is also seen (114). Repeated episodes of disease after environmental exposures to particular substances should raise the suspicion of hypersensitivity pneumonia. It is critical that the nature of the problem be recognized before the development of permanent lung damage. Avoidance of further exposure can lead to an excellent prognosis if the problem is recognized early (115).

In the past, hypersensitivity pneumonitis was considered one of the most clear-cut examples of lung disease mediated by a type III mechanism (85).

This hypothesis was based on the presence of precipitating antibody in the serum to the offending antigen, the similarity of symptoms of hypersensitivity pneumonia to serum sickness, and the time course of the disease after exposure (31, 113). We now recognize that features of both type III immune complex injury and type IV cell-mediated immunologic injury are seen in patients with this disease. The possible role of each mechanism is reviewed in the following paragraph and in the section on type IV diseases.

Several observations argue either for or against a primary pathogenic role of immune complexes in hypersensitivity pneumonitis. First, precipitating antibody which is reactive with the offending antigen is generally found in these patients (116, 117). The presence of precipitins does not, however, necessarily signify disease. For example, they may be present in up to half of exposed but asymptomatic subjects. In addition, they are not present in all patients with overt disease (117, 118). However, patients with more severe disease (119) show higher antibody titers against the offending antigens. In addition, with skin testing, an Arthus-type reaction can occur 4–8 hr after specific antigen challenge, at approximately the same time that bronchial challenge can produce symptoms. However, as suggested by the discussion of type I hypersensitivity, late pulmonary responses after bronchial challenge are not necessarily type III reactions. Other features of hypersensitivity pneumonitis also raise questions about the overall importance of immune complexes in pathogenesis. For example, there are usually no depressions in circulating complement levels during periods of illness that occur naturally or after laboratory exposure to antigen (5). Unfortunately, local complement concentrations or turnover rates in the alveolus or pulmonary interstitium are unknown in this disorder, and it is possible that serum levels may not reflect local events. However, the fact that immunofluorescent studies often fail to demonstrate immune complexes in biopsy specimens of the lung argues that immune complex–induced injury is not a constant feature, especially in chronic forms of this disorder. It is possible, as suggested by Stankus & Salvaggio (116), that immune complex hypersensitivity may be most important in acute forms of illness while cell-mediated immune mechanisms contribute to the pathologic alterations in the more subacute and chronic forms of disease. Thus, biopsies taken within days of acute exposure have shown a vasculitis with neutrophils, C3, IgM, and IgG in vessel walls (120) while chronic disease is associated with interstitial fibrosis (121). Observations suggesting that type IV mechanisms are important in this disease process are reviewed below.

Type IV Reactions

Type IV reactions, also referred to as cell-mediated or delayed hypersensitivity responses, are so named because sensitized T cells can mediate reactions that develop over hours without involvement of either circulating

antibody or complement. These reactions are produced when antigen presents to homologous receptors on the surface of sensitized T cells, which leads to production of soluble factors (lymphokines) that act on other cell types including macrophages. Henney & Newman (122) suggest that T cell–mediated tissue damage may occur by way of two general pathways. First, cytotoxic T cells may directly lyse antigen-bearing cells. This reaction is felt to play a prominent role in allograft rejection as well as in tumor surveillance and in the inhibition of spread of viral infections. The importance of this mechanism in its potential to injure the lung is largely unknown. The other general pathway centers around antigen-initiated production of lymphokines, which leads to activation of a variety of cells (especially macrophages) that effect tissue damage and physiologic alterations. In general, these mediators amplify responses by virtue of their effects on macrophages as well as other cell types, including neutrophils and eosinophils. The lymphokine-associated effects on cells help account for the histologic appearance of some delayed hypersensitivity reactions: Chemotactic lymphokines account for the mononuclear cell infiltration while migration inhibitory factors may explain the persistence of cells in the lesion. The classic example of this type of response is tuberculosis in which cell-mediated immunity against tuberculin accounts for many of the pulmonary histologic findings (4). In this review, two other disease processes in which type IV reactions have been implicated will be discussed: hypersensitivity pneumonitis and sarcoidosis.

HYPERSENSITIVITY PNEUMONITIS Several observations suggest that cell-mediated immune mechanisms contribute to the pathogenesis of hypersensitivity pneumonitis. First, in humans, the histologic abnormalities resemble those of delayed-type hypersensitivity, with a predominance of monocytes and macrophages with granuloma formation (5). As noted above, this may in part reflect the stage of the disease at the time of biopsy. Second, patients with hypersensitivity pneumonitis exhibit phenomena associated with cell-mediated immunity. For example, the antigen that causes the disease can induce stimulation of peripheral blood lymphocytes as assessed by lymphocyte culture techniques (123). In addition, peripheral blood lymphocytes from patients with disease will produce lymphokines such as migration inhibition factor (124). Lymphokine-producing sensitized lymphocytes have been found in lung as well as blood of patients with hypersensitivity pneumonitis (125). While some studies have found that lymphokine production (126) or proliferation of lymphocytes upon exposure to antigen (123) helps to differentiate symptomatic from asymptomatic exposed individuals, it is now clear that these cell-mediated phenomena do not clearly demarcate subjects with and without disease. For example, Moore and co-workers (127) showed that healthy individuals

exposed to pigeons can have bronchoalveolar lymphocytes that proliferate when exposed to antigen.

While it might be argued that these various studies of cell-mediated events are better discriminators of healthy versus symptomatic individuals than are serum precipitins, it is obvious that the events that lead from a healthy to a disease state still elude us. However, recent observations in animals and humans may be helping to delineate some of these factors. Animals given large quantities of antigen via the respiratory tract have not developed chronic interstitial lung disease (128), but antigen administered with concanavalin A, a lectin that stimulates T lymphocytes, produces a granulomatous alveolitis with frank interstitial fibrosis after a 4–8 week chronic exposure (129). If the lectin acted at the level of the helper T cell, the suppressive forces that normally dominate immunoregulation might have been overcome. Moore and associates (130) have made other observations that suggest hypersensitivity pneumonitis represents an imbalance between helper and suppressive functions of T cells. These investigators found that a strain of mice, resistent to chronic pulmonary granulomatous inflammation induced by intravenous administration of killed BCG-emulsion, would develop this disease if treated with cyclophosphamide before challenge. The hypothesis that cyclophosphamide worked via suppressor T cells was strengthened by the fact that the effects of cyclophosphamide were inhibited by provision of syngeneic whole or purified spleen T cells from mice injected seven days earlier with the BCG-emulsion. In a nonhuman primate model of pigeon breeder's disease, Keller and co-workers (131) found, in two monkeys that did not develop the picture of hypersensitivity lung disease, a population of T-suppressor cells that in animals who developed disease were missing or nonfunctional. The investigators reported low-dose total body irradiation to inactivate T-suppressor cells led to the development of pulmonary symptoms after challenge in the two previously nonresponsive animals. While these observations are obviously interesting, further studies to delineate the role of these immunoregulatory cells in animals and humans with this disease are needed.

SARCOIDOSIS Sarcoidosis is a systemic granulomatous disease of unknown etiology. Almost all organs of the body can be affected, but the respiratory tract is most commonly involved and is the organ most closely associated with morbidity and mortality. The disease is usually insidious in onset with symptoms of dry cough, dyspnea, and exercise intolerance referrable to the lung involvement (95). Examination of the chest often discloses dry rales at the bases, and the chest X ray can show hilar adenopathy and/or parenchymal changes (132). In general, assessment of lung function shows a restrictive pattern, although airflow limitation, presumably because of granulomas in airway walls, has been described (133). The diagnosis of

pulmonary sarcoid rests on the demonstration of noncaseating granulomas in intrathoracic tissue.

The concepts of pathogenesis in sarcoidosis have recently changed. Part of this change is due to study of bronchoalveolar lavage fluid in untreated patients with active pulmonary disease; this reveals an alveolitis dominated by lymphocytes and macrophages (134–138). We do not understand the events that lead to T-cell and alveolar macrophage activation and to mediator release. No specific antigen has been associated with the disease. One theory is that sarcoidosis occurs in patients whose immune system overreacts to antigens in general by not properly modulating processes that follow antigen recognition (95).

The lymphocyte population that characterizes pulmonary sarcoidosis is the helper T cell. The T-cell population of the normal lung shows a T-helper to T-suppressor ratio of 1.8 : 1, but increases to 10 : 1 in active sarcoid (138, 139). In addition to being present in larger numbers than normal, the T cells are activated and release mediators important for granuloma formation (95, 135), including a monocyte chemotactic factor and migration inhibitory factor. The opposite pattern of T cell ratios is present in the blood of patients with active sarcoid. A lymphopenia with a helper : suppressor ratio of 0.8 : 1 (139) instead of 1.8 : 1 is found. In addition, while lung T cells spontaneously release mediators important in monocyte movement and B cell activation, peripheral blood T cells do not (135, 137). Thus, the peripheral blood does not reflect changes in the lung that may be of pathogenic significance.

The number of alveolar macrophages present in lavages of patients with active sarcoidosis is increased, but not to the same extent as the T cells. Recently, Hunninghake (140) has reported that in vitro, alveolar macrophages from sarcoid patients with high-intensity alveolitis release significantly greater amounts of lymphocyte-activating factor (interleukin-1) than macrophages from normal subjects, patients with idiopathic pulmonary fibrosis, or sarcoid patients with low-intensity alveolitis. One interpretation of these observations is that alveolar macrophages have a central role in this disease by stimulating lung T cell replication, thus modulating lung lymphocyte function. However, while alveolar macrophages may modulate the proliferation of lung T cells, T cells can recruit and activate mononuclear phagocytes. Thus, basic questions remain: What initiates the disease, and does that agent(s) work primarily through the alveolar macrophage or lung T cell?

CONCLUSION

In the lung as in other organs, presentation of antigen does not engage just one facet of the immune system. Indeed, all components are probably

involved. In addition to the types of immunologic reactions initially described by Coombs & Gell (1), it is also apparent that certain antigens, such as those that cause hypersensitivity pneumonitis, may initiate inflammation through such other mechanisms as the direct activation of the complement system (141). Thus, when we are confronted with a disease where tissue inflammation suggests that hypersensitivity reactions have occurred, it is exceedingly difficult at times to sort out pathogenesis. On top of this, we are dealing with reactions that should be beneficial to the host; but because of the nature of the antigen, the genetics of the host, an altered immunologic response, and/or other factors not currently understood, these reactions have led to tissue injury. While this may appear to be an unsolvable puzzle for many diseases, it is pertinent to recall the progress made in the last two decades that has increased our understanding and led to modification of our thoughts about hypersensitivity reactions. Through basic research as well as studies in animal models and man, clearer pictures of basic immunologic reactions as well as mechanisms of disease will continue to emerge.

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CONTENTS

Ann. Rev. Immunol. 1985. 3:87–108

PATHOGENESIS OF AUTOIMMUNITY IN PEMPHIGUS

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INTRODUCTION

Adhesion of cells is necessary for a number of cell functions, such as organization of tissues and cell motility. In addition, the adhesive properties of some tumor cells are likely to be important in metastasis. Alteration of cellular adhesive properties can result in severe pathology. An example of this is the blistering autoimmune disease, pemphigus, in which there is an alteration of cellular adhesion and, ultimately, a loss of cohesion of the epithelium of skin and mucous membranes.

In this review we have described the clinical features of pemphigus and reviewed the evidence that pemphigus is a disease mediated by the immune system. Studies on the pathogenesis of pemphigus are presented along with a hypothesis for the molecular mechanism involved. We have been aided in these investigations by the fact that pemphigus, unlike other autoimmune

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diseases, is basically a single-system disease with autoantibodies of highly restricted specificity. Our results provide evidence for autoantibody-mediated regulation of the disease by stimulation of the production of a proteolytic enzyme, specifically plasminogen activator. The potential importance of antibody for the disruption of cellular adhesion in the absence of cytotoxic effects is evident from these studies.

CLINICAL FEATURES OF PEMPHIGUS

Pemphigus is a potentially fatal disease manifested by severe blistering of the skin and mucous membranes (1), and characterized by the presence of autoantibodies directed against the surface of cells of stratified squamous epithelia (2). While pemphigus may occur at any age, its most common onset is in the fourth, fifth and sixth decades. We have no direct evidence that pemphigus is a genetic disease, but the observation that it occurs more frequently in people of Jewish or Mediterranean heritage (3) and appears within families (4, 5) indicates that there may be a genetic component to its development. Based on clinical presentation, histopathology, and immunopathology, several types of pemphigus can be distinguished and grouped into two major categories, pemphigus vulgaris and pemphigus foliaceus.

Pemphigus Vulgaris

Pemphigus vulgaris has been reported to be the most common form of the disease (6–8); however, over the last eight years at Duke we have seen approximately equal numbers of patients with vulgaris and foliaceus (D. Tashjian, personal communication). The cutaneous lesions of pemphigus vulgaris are characterized by flaccid, weeping bullae that leave geometric erosions and large areas of denuded skin. Blisters are most prominent on the trunk and in the intertriginous areas. Skin that appears normal may be induced to form blisters upon application of lateral pressure (Nikolsky's sign). This important physical finding indicates that epidermal cohesion is abnormal even in skin without lesions. Pemphigus vulgaris commonly involves the mucous membranes, primarily oral and genital, and up to 60% of patients present initially with oral lesions (6, 7). Histopathologically, pemphigus vulgaris is characterized by suprabasal, intraepidermal bullae formation (Figure 1A). Some epidermal cells round up and lose cohesion, a phenomenon termed acantholysis (9, 10). Basal cells remain attached to the dermis in the basement membrane zone and may appear as a "row of tombstones" (1). A sparse perivascular infiltrate of mixed inflammatory cells may occur in areas of acantholysis (11).

A rare variant of pemphigus vulgaris is pemphigus vegetans. In the early stages of this disease lesions are identical to those of pemphigus vulgaris;

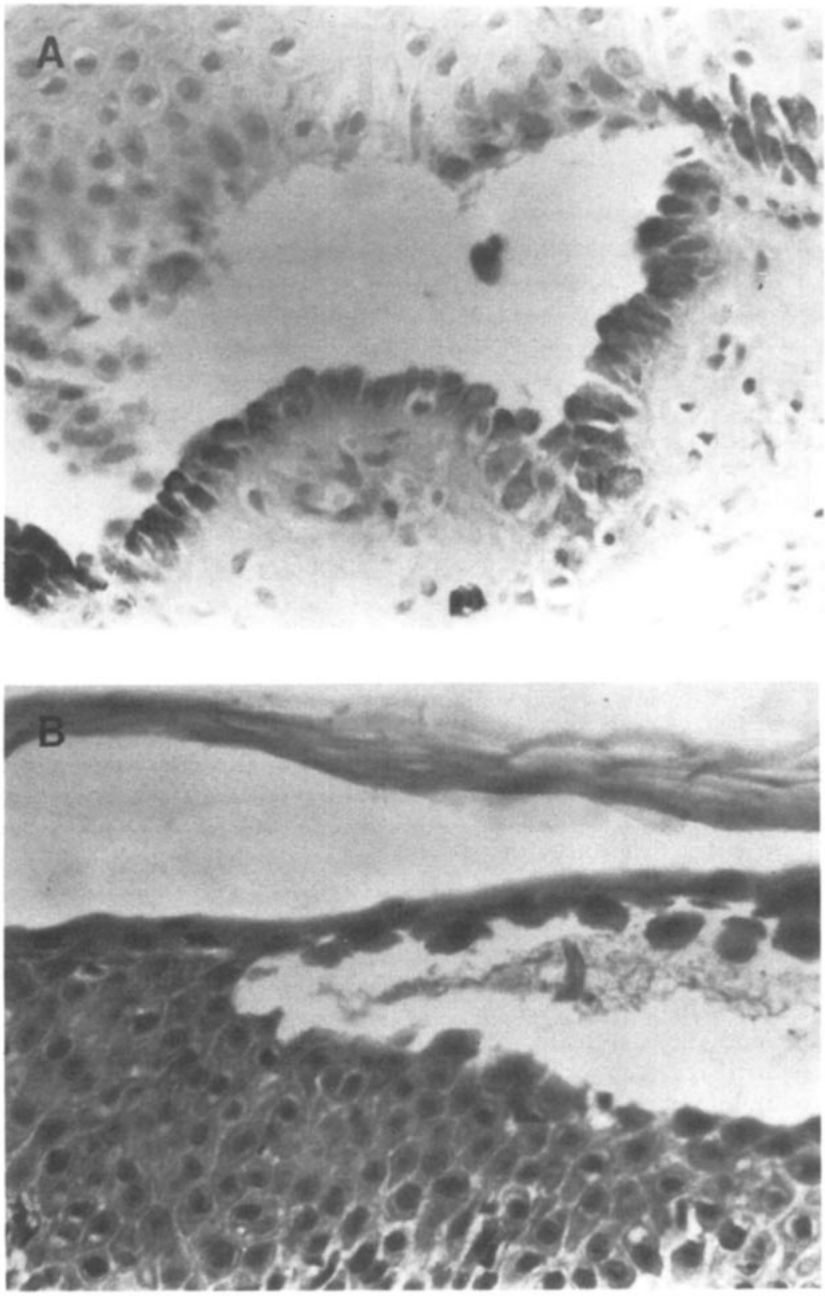

Figure 1 Photomicrographs of hematoxylin and eosin-stained sections from lesional biopsies of patients with (*A*) pemphigus vulgaris and (*B*) pemphigus foliaceus.

during healing, however, hypertrophic, verrucoid granulations arise at the site of ruptured bullae. Pustules may be found at the periphery of these vegetating lesions. Skin biopsies often reveal intraepidermal accumulations of eosinophils (12).

Pemphigus Foliaceus

A less severe form of pemphigus, pemphigus foliaceus is characterized by shallow erosions, scales, and crusting. Cutaneous lesions frequently develop on the scalp, face, chest, and back. Bullae are fragile and rupture easily; consequently, patients may present without frank bullae. Mucous membrane involvement is uncommon in pemphigus foliaceus. In contrast to that of pemphigus vulgaris, the histopathology of pemphigus foliaceus lesions reveals acantholysis high in the epidermis, at or near the granular layer (Figure 1B). Neutrophils may be present within the blister (13, 14).

An interesting variant of pemphigus foliaceus, Brazilian pemphigus or "fogo selvagem" (wildfire) is more appropriately called pemphigus foliaceus endemicus (PFE). While PFE cannot be distinguished histopathologically from pemphigus foliaceus, it is readily distinguished by its epidemiology (15). The epidemiologic features include an endemic occurrence in certain rural regions of South America (particularly Brazil) generally affecting children, adolescents, and young adults. Frequently, multiple cases occur within a single family. The distribution of the disease has suggested the involvement of an arthropod vector but no evidence for this has been found. Investigators have attempted to prove a viral etiology, but the studies are inconclusive.

Another variant of pemphigus foliaceus is pemphigus erythematosus (Sinear-Usher Syndrome), thought to be an overlap syndrome of pemphigus and lupus erythematosus (16).

Therapy

Before the use of systemic corticosteroids, pemphigus resulted in mortality rates of 70–100% (17). Since the introduction of steroids, mortality from pemphigus has reportedly dropped to 15–44%. Combination therapy of systemic corticosteroids and immunosuppressive agents is associated with a mortality rate of 10–20% (18–21). Steroids are the most widely used therapeutic agents and can be highly effective in some cases; however, the chronic use of high-dose steroids produces serious side effects and contributes to the mortality rates. Suprapharmacologic doses of methylprednisolone (pulse therapy) have been used with some success (22). Treatment of pemphigus with immunosuppressive agents (particularly azathioprine, cyclophosphamide, and methotrexate) is promising, and successful therapy has been reported with gold, dapsone, sulfapyridine, and

plasmapheresis (reviewed in 21). Unfortunately, no extensive controlled studies have been designed to compare the different types of therapy.

PEMPHIGUS AS AN AUTOIMMUNE DISEASE

Immunopathology

IMMUNOFLUORESCENCE Beutner & Jordan (2) provided the first clue to the etiology of pemphigus in 1964 when they demonstrated that serum from pemphigus patients contained autoantibodies that bound to an intercellular substance (ICS) of skin and mucosa. Subsequently, skin biopsies revealed in vivo deposition of autoantibodies in the epidermis of pemphigus patients (23). It is generally agreed that the antigen or antigens to which the autoantibodies bind are found on the surface of cells of stratified squamous epithelium. In general, there is a correlation between circulating antibody titers and disease activity, but this may vary with the source of normal tissue used to screen patient serum (11).

Direct immunofluorescence provides a diagnostic test for pemphigus. Perilesional skin biopsies show deposition of immunoglobulin, primarily IgG, within the epidermis in a characteristic cell-surface pattern. In pemphigus vulgaris patients, the IgG is deposited throughout the entire epidermis. In contrast, some patients with pemphigus foliaceus have IgG deposited only in the upper layers of the epidermis (24); however, other patients have IgG bound throughout the epidermis. Interestingly, circulating autoantibodies in pemphigus foliaceus bind to all layers of normal epithelium in indirect immunofluorescence studies. Stanley et al (25) have begun to examine these paradoxes. These investigators have isolated a unique antigen to which pemphigus vulgaris antibodies bind which is distinct from the antigen(s) detected by pemphigus foliaceus antibodies.

ULTRASTRUCTURAL STUDIES Early electron microscopic studies of pemphigus vulgaris (26, 27) produced conflicting interpretations of the earliest pathologic changes in pemphigus. In 1967, utilizing the newly available data on the binding of autoantibodies to the ICS, Hashimoto & Lever reexamined the ultrastructural morphology of lesional skin (28, 29). They found that where the epidermal cells first lost cohesion there was partial or complete dissolution of the intercellular substance, and this preceded disintegration of desmosomes. Hashimoto & Lever also suggested that the "tombstone" row arrangement of basal cells was produced by dissolution of the ICS around basal cells, with the base of the basal cells remaining attached to the dermo-epidermal junction.

ROLE OF COMPLEMENT The role of complement in pemphigus has not been elucidated. In early acantholytic lesions, C3 as well as the classical pathway

components, C1q and C4, have been detected (30–32). Several alternative pathway factors, such as properdin and factor B, have also been observed, although less frequently. In contrast, in the skin of pemphigus vulgaris patients which appears clinically normal, immunoglobulin but not complement can be detected by immunofluorescent techniques. This same normal-appearing skin demonstrates abnormal mechanical fragility and it can be induced to blister by mechanical pressure (Nikolsky's sign). These observations suggest that complement is not involved in the development of acantholysis. In vitro studies have tended to rule out a primary role for complement in induction of acantholysis (discussed below). Recently, using an in vitro assay to assess loss of adhesion of mouse epidermal cells, Kawana et al reported that the presence of complement with pemphigus antibody enhanced the loss of adhesion of cells beyond the loss seen with antibody alone (33). They suggested that this is evidence of a role for complement in the induction of acantholysis; however, their results can explained as a loss of cell adhesion following cytotoxicity induced by antibody and complement.

PATHOGENICITY OF AUTOANTIBODY While the presence of an autoantibody directed against squamous epithelia is a characteristic finding in pemphigus, it does not prove that antibody plays a role in the acantholytic process. The following three lines of evidence do support a role for antibody in the induction of acantholysis in pemphigus: (*a*) correlation of antibody titers with disease activity (34, 35); (*b*) induction of acantholytic lesions in the skin of neonatal mice (36) or in grafts of human oral mucosa on nude mice (37) following passive transfer of patient IgG; and (*c*) induction of acantholytic lesions in explants of normal human skin incubated with pemphigus IgG (38, 39).

Pemphigus can also be induced by drug therapy (primarily penicillamine) or physical factors (ultraviolet light and infrared and ionizing radiation) (40). We have recently reported that penicillamine-induced pemphigus and spontaneous pemphigus share common pathophysiological processes in blister formation (41).

Association with Other Immune Disorders

Thirty patients have been reported with pemphigus associated with myasthenia gravis or thymoma. Some of these patients have the entire triad of pemphigus, myasthenia gravis, and thymoma (reviewed in 40). Interestingly, reports suggest that pemphigus antibodies bind to Hassall's bodies of the thymus (42, 43). There is increasing evidence that thymus and epidermis share an interactive relationship with T lymphocytes (44) and share certain tissue-restricted cell-surface and cytoplasmic differentiation

markers (45, 46). Investigation of this relationship may provide information for understanding the relationship between pemphigus, thymoma, and myasthenia gravis (47).

Pemphigus erythematosus (Sinear-Usher Syndrome) is an overlap syndrome with features of both pemphigus and lupus erythematosus: the butterfly distribution of lesions on the face, immunoglobulin deposition at the dermo-epidermal junction, and antinuclear antibodies (48).

Association with Antigens of the Major Histocompatibility Complex (MHC)

A number of studies show association of HLA antigens with pemphigus, primarily in Jewish and in Japanese patients, significantly in HLA-A13 (49), HLA-A10 (50, 51), HLA-A26 (a subgroup of A10) (52, 53), HLA-BW28 (54), and HLA-DR4 (53). Of these the most striking association is with DR4 which, according to Park et al, carried a relative risk factor of 31.5 in the population they studied (53).

IN VITRO STUDIES OF PATHOGENESIS

As early as 1956 (55) Bellone and Leone reported acantholysis in vitro in normal human skin explants incubated with pemphigus patient serum; but because the publication appeared in Italian, it was not widely read. Finally in 1974 (56) and 1977 (38), in more widely read publications, Michel & Ko reported evidence supporting a role for antibodies in the acantholytic event.

Michel & Ko incubated fresh explants of human skin in organ culture with whole, undiluted serum from either pemphigus vulgaris or pemphigus foliaceus patients (38). Using immunofluorescence techniques, they showed that by 6–12 hr, IgG was deposited in the intercellular spaces of the epidermis of the explant in a pattern consistent with the deposition of IgG in vivo in pemphigus patients. No IgG binding was detected in skin explants incubated with control human sera. Light microscopic observation of skin explants incubated with pemphigus sera showed histologic changes that mimicked the pathology of pemphigus vulgaris. By 24–72 hr, suprabasilar separation of the epidermis occurred and acantholytic cells were observed. No acantholysis was observed in explants incubated with control sera. All sera were heated to destroy complement activity, and complement was not detectable in explants by immunofluorescence. These results were confirmed by Chorzelski and his colleagues using monkey skin (57).

The organ culture model system made it possible to examine, at a molecular level, the pathophysiology of pemphigus. This model remains the

best and most relevant one in which to test new hypotheses for the mechanism of pemphigus.

Schiltz & Michel (39) provided experimental evidence that the anti-ICS autoantibody of pemphigus patients was the serum component responsible for inducing acantholysis. Preparations of IgG (ethanol precipitates, 30–40 mg/ml) were incubated with normal human skin explants. Epidermal acantholysis, with the presence of acantholytic cells was observed by 72 hr. No acantholysis was seen in control explants incubated with normal human IgG. IgG preparations were heated to 58°C for 1 hr without loss of the ability to induce acantholysis; this suggests that complement was not necessary.

Michel and his colleagues were able to confirm, on an ultrastructural level (58), the marked similarities between the acantholysis seen in organ cultures exposed to pemphigus IgG and that observed by Hashimoto & Lever in the lesions of pemphigus patients (28).

Enzymatic Mechanism

The concept of an enzymatic mechanism for acantholysis is an old one and has been suggested for a number of in vitro models of acantholysis. One of the most convincing studies was published in the late 1950s by Stoughton & Bagatell (59), who reported the enzymatic nature of acantholysis induced by cantharidin (a product of the beetle *Canthous vesicatoria*). They concluded that the enzyme induced following treatment with cantharidin was produced by the skin and was heat labile, sulfhydryl dependent, and inhibited by hydrocortisone and diisopropyl fluorophosphate. The acantholysis induced by cantharidin was almost indistinguishable from that seen in pemphigus vulgaris.

In 1978, our laboratory presented evidence for an enzymatic mechanism in the induction of acantholysis by pemphigus IgG (60). The original observations were made using cultured neonatal mouse epidermal cells, which were shown by immunofluorescence to bind pemphigus IgG. The cells were radiolabeled with ^{3}H-thymidine and incubated with IgG preparations (ammonium sulfate fractionation) from pemphigus vulgaris patients, patients with pemphigoid (another bullous skin disease), or normal individuals. After 8, 24, and 48 hr of incubation, the cells were washed gently with overlying medium, and, as a method for counting detached cells, this supernatant containing detached cells was evaluated for acid-precipitable radioactivity. After removal of detached cells, the cells remaining adherent to the dish were also enumerated by measuring radioactivity. The results showed that incubation of cultured mouse epidermal cells with IgG preparations from pemphigus patients induced

detachment of 60% of cells from the culture dish. Incubation with IgG from a bullous pemphigoid patient induced only slightly more detachment than the background levels established with normal human IgG preparations (less than 20% of cells detached). Viability studies indicated that pemphigus antibody was not cytotoxic. Two proteinase inhibitors, soybean trypsin inhibitor and alpha$_2$-macroglobulin, blocked the cell detachment induced by pemphigus antibody. Results in this model system led to the hypothesis that binding of pemphigus autoantibody to epidermal cells stimulated an enzyme or enzymes, which were then responsible for loss of epidermal cell adhesion.

In an effort to evaluate at a molecular level the pathologic events of pemphigus, Schiltz et al (61) performed experiments using epidermal cell suspensions. These suspensions were prepared using trypsin and incubated in tissue-culture medium containing IgG preparations (ethanol precipitates, 20–40 mg/ml) from either pemphigus or normal serum. After 18 hr of incubation with pemphigus IgG, 75% of the cells were dead, as judged by uptake of vital dye, compared with 14% dead in cell suspensions incubated with normal IgG. Unfortunately, the cell suspension culture system may have led Schiltz and co-workers to overestimate the cytotoxic nature of pemphigus antibodies in the absence of complement. Rheinwald has shown that human epidermal cells placed in suspension culture rapidly undergo terminal differentiation and die (62).

Subsequently, Schiltz and his colleagues (63) reported that they could recover a proteolytic enzyme in the culture medium of skin explants incubated with pemphigus IgG, but not from those incubated with normal IgG. Using a radiolabeled insoluble epidermal preparation as substrate, they observed a pH optimum for the enzyme at pH 6.5. Maximal enzyme activity coincided with the time of onset of acantholysis. Lysosomal hydrolases were not detected in the culture medium, suggesting that the enzyme causing acantholysis was not of lysosomal origin. Following removal of pemphigus IgG, the conditioned medium caused acantholysis in fresh explants. Based on these organ culture data, they hypothesized that interaction of pemphigus autoantibody with the epidermal cell surface induces synthesis or activation of what they termed "pemphigus acantholytic factor" (PAF), perhaps a nonlysosomal proteinase that would cause loss of cellular adhesion and acantholysis. Because they were unable to inhibit pemphigus antibody-induced acantholysis in vitro using soybean trypsin inhibitor (64), they did not consider it likely that PAF was a serine-class proteinase.

In 1981, Morioka et al (65) reported that soybean trypsin inhibitor and pepstatin A inhibited acantholysis induced in normal skin by pemphigus

antibodies. These results confirmed our conclusion (60) that a serine-class proteinase was involved and suggested the involvement of carboxyl proteinase(s) as well.

The Role of Plasminogen Activator in Blister Formation

In 1981, Becker et al (66) reported that production of plasminogen activator (PA) by two different cell lines (mouse fibroblast and pig kidney) could be stimulated by rabbit antibodies raised against the cells. Their results suggested to us the possibility that plasminogen activator might be the serine proteinase stimulated in epidermal cells following the binding of pemphigus autoantibody. We have reported evidence to support this hypothesis (67).

Plasminogen activators are serine proteinases that catalyze the conversion of plasminogen to plasmin. Recently the role of plasminogen activators in the regulation of localized proteolysis within the microenvironment of cells has received much attention. A wide variety of normal vertebrate adult and embryonic cells produce PA under certain circumstances, and this production is highly regulated (for review see references 68–70). PA is of critical importance in tissue remodeling and cell migration.

Confluent primary cultures of neonatal human epidermal cells, cultured by the method of Rheinwald & Green (71), were incubated in tissue culture medium either (*a*) containing IgG (affinity purified, plasminogen-free) from pemphigus patients or normal controls, or (*b*) without IgG. Following a 24-hr incubation, conditioned medium was collected. The cells were then washed and lysed using the detergent Triton X-100. We examined medium and cell lysates for secreted and cell-associated PA, respectively. The incubation of epidermal cells with pemphigus foliaceus IgG resulted in a 10-fold increase in extracellular PA and a 5-fold increase in cell-associated PA. The stimulation of both extracellular and cell-associated PA was maximal at 2 mg/ml. At higher concentrations of IgG, less stimulation was observed. Incubation of epidermal cells with pemphigus vulgaris IgG resulted in a 7-fold increase in extracellular PA and a 6-fold increase in cell-associated PA. The stimulation of both extracellular and cell-associated PA by pemphigus vulgaris IgG reached a maximum at 0.5 mg/ml.

Kinetic experiments indicated that stimulation by antibody of cell-associated PA could be detected as early as 6 hr and reached plateau levels by 12 hr. Stimulation of extracellular PA levels was not detected before 12 hr but continued to rise beyond a 24-hr incubation with antibody.

We have documented in previous studies that pemphigus antibody in the absence of complement is not cytotoxic for human or mouse epidermal cells. It was important to determine if the increased enzyme activity we observed might be related to previously undetected cell damage or to

regurgitation of lysosomal contents during endocytosis. We assayed the conditioned medium for Cathepsin D as a marker for the release of lysosomal enzymes. Epidermal cells were incubated for 24 hr with IgG preparations at various concentrations up to 8 mg/ml. The medium was then tested for the ability to hydrolyze ^{3}H-hemoglobin. Medium from cells incubated with 8 mg/ml IgG had slightly higher hydrolytic activity than that from cells incubated without IgG. Medium collected from cells incubated with 0.5 mg/ml IgG, which is the optimum for PA stimulation, hydrolyzed hemoglobin to the same extent as did that collected from cells incubated without IgG. We concluded that the antibody-mediated stimulation of PA activity was not related to cell damage and was not accompanied by a generalized secretion of lysosomal enzymes.

In order to determine if stimulation of PA activity required protein synthesis, we incubated epidermal cells for 12 hr with pemphigus foliaceus IgG in the presence of various concentrations of cycloheximide. Twenty μg/ml cycloheximide inhibited extracellular and cell-associated PA activity by 95% and 90%, respectively. This concentration of cycloheximide inhibited incorporation of ^{35}S-methionine into proteins by 82%. These data confirm that intact, metabolically active cells are required for the stimulation of PA by pemphigus IgG.

The stimulation of PA production by cells treated with lectins and phorbol esters, and by thioglycollate-stimulated macrophages, is inhibited by steroids (72–74). Considering these reports, and the fact that most pemphigus patients respond to high doses of steroids with a relatively rapid reduction in the formation of new blisters, we examined dexamethasone for its ability to inhibit PA stimulation by antibody. Epidermal cells were incubated for 24 hr in the presence of pemphigus foliaceus IgG and varying concentrations of dexamethasone. At 10^{-10} M, extracellular PA activity was inhibited by 60%. At 10^{-8} M, 95% inhibition of antibody-induced PA was achieved (67). The inhibitory concentrations of dexamethasone approximate those achieved in the clinical treatment of pemphigus. Schiltz et al (64) reported that hydrocortisone could not prevent acantholysis in human skin explants cultured with pemphigus IgG; Swanson & Dahl (75), however, found that methylprednisolone could prevent acantholysis in human skin explants cultured with pemphigus IgG when methlyprednisolone was added 24 hr before initiating incubation with pemphigus IgG. The discrepancy may be explained as a difference in the inhibitory activity of the particular steroid derivative and the potential need for preincubation with steroid. We have recently reported (76) that at 10^{-7} M methylprednisolone significantly inhibited the PA production stimulated by pemphigus antibody, while hydrocortisone had no effect at that concentration. At 10^{-5} M both methylprednisolone and hydrocortisone inhibited

PA production. Neither dapsone nor gold salts inhibited PA production induced by pemphigus antibody. The mechanism of inhibition of PA production by steroids in this system is not known, but corticosteroids have been reported to induce inhibitors of PA (77, 78). Anhalt et al (79) were unable to inhibit experimental pemphigus in newborn mice with corticosteroids; interpretation of these experiments may be difficult, however, because of the difference in steroid responsivity of the different species.

Two major types of human plasminogen activators are distinguished biochemically and immunologically: "urokinase type" (M_r 52,000–55,000) and "tissue type" (M_r 60,000–74,000) (80). In order to determine which type of PA was produced by human epidermal cells, we analyzed samples of conditioned medium from human epidermal cell cultures for PA activity following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic profile of enzymatic activity indicated that the major peak of PA activity secreted by cultured human epidermal cells is of M_r 55,000, suggesting that it is a urokinase-type enzyme. A trailing of enzymatic activity was seen at higher molecular weights. It was important to determine if pemphigus antibody stimulated the production of more urokinase-type PA or induced production of the tissue activator. Accordingly, conditioned medium was collected from cells incubated with pemphigus IgG and the PA activity analyzed following SDS-PAGE. The electrophoretic profile of conditioned medium from cells treated with pemphigus vulgaris IgG or pemphigus foliaceus IgG did not differ from that of untreated cells. These data suggest that pemphigus antibody stimulates the production of plasminogen activators normally produced by cultured human epidermal cells.

To investigate the role of antibody-induced increases in plasminogen activator on the loss of epidermal cohesion, we performed skin explant experiments in the presence or absence of pemphigus IgG, human plasminogen, and a variety of proteinase inhibitors. Figure 2 presents the striking results we obtained using an IgG preparation (affinity purified on protein A-Sepharose, and plasminogen-free) from a pemphigus foliaceus patient. No acantholysis was seen in normal skin explants incubated with IgG from the patient (4 mg/ml) (Figure 2A) or human plasminogen (200 μg/ml) alone (Figure 2B). However, when human plasminogen was included along with the patient's IgG (Figure 2C), acantholysis was consistently observed in the upper epidermis, and was indistinguishable from the patient's lesional biopsy. When aprotinin (which inhibits plasmin but not plasminogen activator) was included with pemphigus foliaceous IgG and plasminogen, production of acantholysis was inhibited (Figure 2D). We obtained identical results using lima bean trypsin inhibitor (LBTI), which also inhibits plasmin but not plasminogen activator.

Normal human skin, incubated for 48 hr with IgG from a pemphigus vulgaris patient (4 mg/ml), demonstrated histologic changes resembling pemphigus vulgaris (Figure 3A). The inclusion of human plasminogen (320 μg/ml) appeared to hasten the onset of these changes and to increase the extent of suprabasilar acantholysis in the explant (Figure 3B); however, we

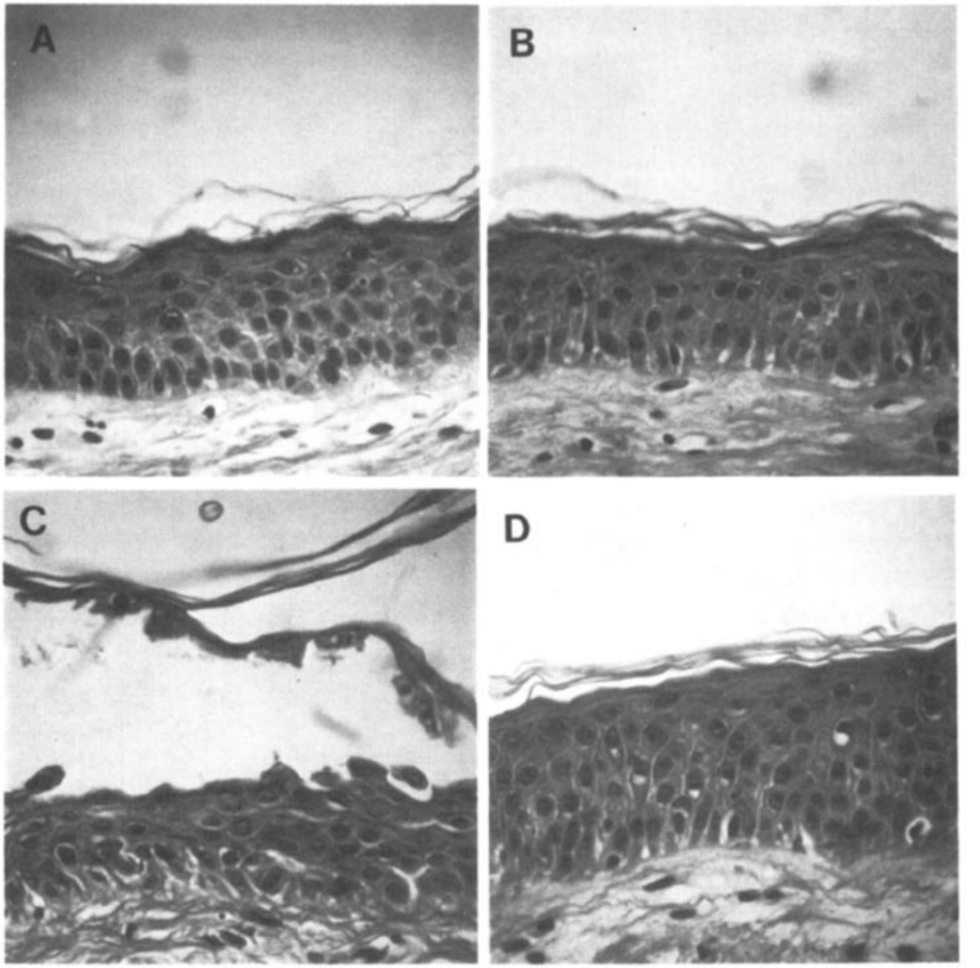

Figure 2 Explants of normal human skin incubated 48 hr with (*A*) pemphigus foliaceus IgG (4 mg/ml), (*B*) plasminogen (200 μg/ml), (*C*) pemphigus foliaceus IgG (4 mg/ml) plus plasminogen (200 μg/ml), (*D*) pemphigus foliaceus IgG (4 mg/ml), plasminogen (200 μg/ml) plus aprotinin (total of 60 trypsin inhibitory units added in doses of 20 units at time 0, 20 hr, and 30 hr).

did not demonstrate a strict dependence upon exogenously added plasminogen. Recently Isserof & Rifkin (81) reported that plasminogen is present in the basal layer of human epidermis. Endogenous levels of plasminogen may therefore be sufficient to result in acantholysis deep in the epidermis. No acantholysis was observed in explants incubated with pemphigus vulgaris IgG and aprotinin or LBTI in the presence or absence of plasminogen

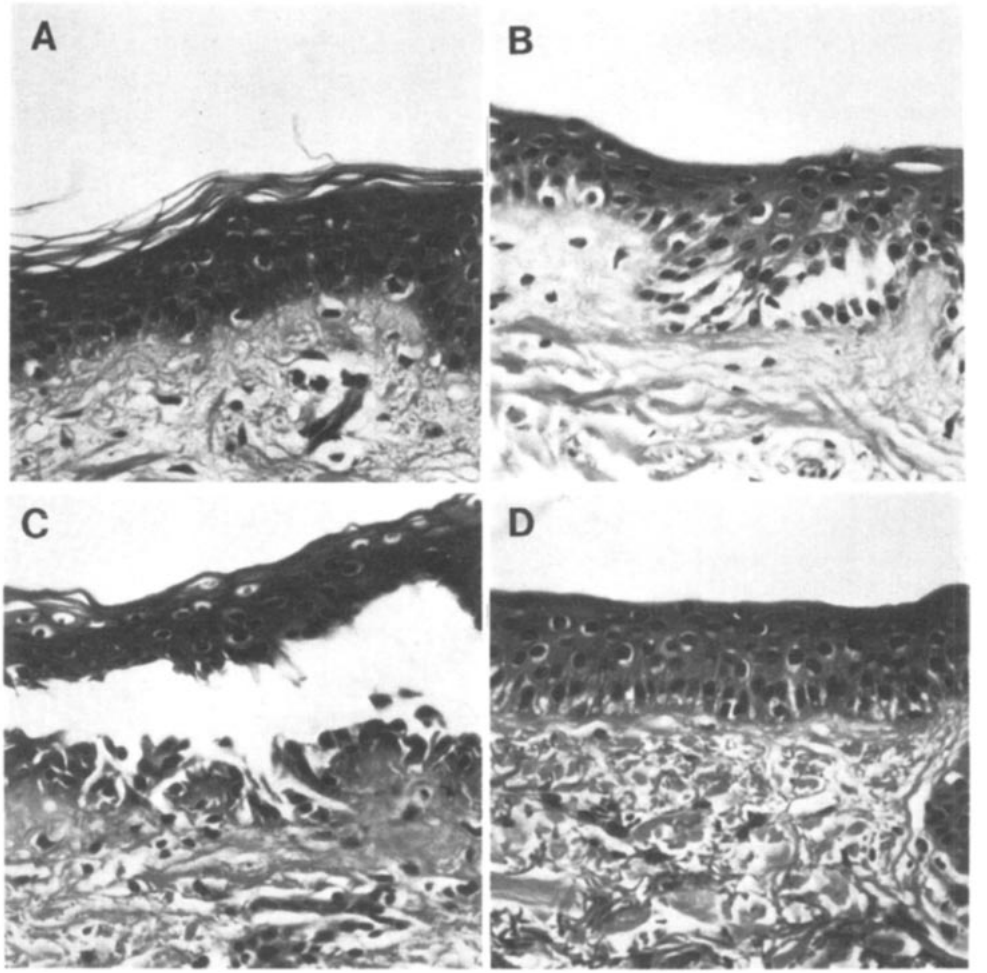

Figure 3 Explants of normal human skin incubated 48 hr with (*A*) pemphigus vulgaris IgG (4 mg/ml), (*B*) plasminogen (320 μg/ml), (*C*) pemphigus vulgaris IgG (4 mg/ml) plus plasminogen (320 μg/ml), (*D*) pemphigus vulgaris IgG (4 mg/ml), plasminogen (320 μg/ml) plus aprotinin (total of 60 trypsin inhibitory units).

(Figure 3C). In addition, we have recently shown that acantholysis was totally inhibited if rabbit anti-urokinase antibody was included in the incubation medium of organ cultures. The same anti-urokinase antibody completely inhibited epidermal cell PA activity (S. Morioka, P. J. Jensen, G. S. Lazarus, unpublished results).

Recent experiments indicate that epidermal PA may exist as a zymogen, which is not sensitive to inhibitors of serine-class proteinases and can be activated by limited proteolytic cleavage (P. J. Jensen, S. Morioka, G. S. Lazarus, unpublished results). In further studies on the regulation of PA in epidermis, we are investigating the presence of endogenous PA inhibitors in epidermis. Several peaks of inhibitory activity have been detected after fractionation of epidermal extracts on sephacryl S-200 (S. Morioka, P. J. Jensen, G. S. Lazarus, unpublished results).

Our work provides evidence for autoantibody-mediated regulation of a specific disease process by activation of proteolytic enzymes. Our data support the following as the molecular mechanism for the autoimmune disease pemphigus (Figure 4): Binding of pemphigus autoantibody to the surface of human epidermal cells stimulates the production of plasminogen activator. In the presence of plasminogen, the increased levels of PA result in activation of plasminogen to plasmin. Plasmin is then responsible for loss of cellular adhesion, probably at least in part by degradation of cell-surface molecules responsible for cell-to-cell adhesion within the epidermis.

The antibody-induced loss of epidermal cohesion in the explants incubated with pemphigus foliaceus IgG was dependent upon the addition of plasminogen, and the loss of adhesion was inhibited by aprotinin or lima bean trypsin inhibitor, indicating that plasmin is the active enzyme in producing acantholysis. It is likely that the IgG preparations as well as serum supplements, which were used by other investigators to produce acantholysis in skin explants, contained plasminogen. We have found that

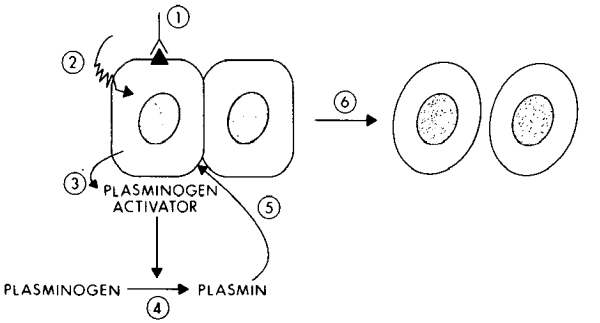

Figure 4 Model for the pathogenesis of pemphigus.

IgG prepared by either ammonium sulfate precipitation or protein A-Sepharose affinity chromatography was contaminated with plasminogen (82). The data presented here were obtained using IgG preparations that had been passed over lysine-Sepharose columns to remove plasminogen.

The introduction of corticosteroid therapy greatly reduced the significant mortality from pemphigus. In high doses corticosteroids rapidly and dramatically reverse blister formation in pemphigus. We observed that suprapharmacologic methylprednisolone (pulse therapy) resulted in clearing of blisters within 48 hr (G. S. Lazarus, unpublished results). Pemphigus antibody titers, however, drop significantly only after 2–4 months of steroid therapy, and some patients become clinically free of disease while still possessing an antibody titer (35). It is conceivable that corticosteroids act, at least in part, by inhibiting antibody stimulation of PA or by inducing an inhibitor of PA.

Antigen Specificity of Pemphigus Autoantibody

A number of studies designed to elucidate the biochemical characteristics of the antigen(s) recognized by pemphigus antibody were recently reviewed by Labib et al (83). Use of different techniques to isolate antigens from a variety of tissue sources has led to conflicting conclusions. Most recently, radioimmunoprecipitation followed by SDS-PAGE and SDS-PAGE followed by Western blot techniques have been applied to the pemphigus system.

Stanley (84) characterized the pemphigus antigen extracted from cultured human epidermal cells and from a transformed mouse epidermal cell line. The cells were labeled with ^{14}C-glucosamine, and extracted with a nonionic detergent. The extract was subjected to immunoprecipitation with sera from a number of pemphigus patients, and precipitates were analyzed by SDS-PAGE. Five of seven pemphigus sera specifically precipitated a glycoprotein antigen of M_r 130,000. Recently, Stanley et al (25) used the same techniques to address the question of whether the antigens detected by pemphigus vulgaris sera differed from those detected by pemphigus foliaceus sera. Under reducing conditions, 12 of 12 pemphigus vulgaris sera precipitated 2 bands of protein with M_r 130,000 and M_r 80,000, Under nonreducing conditions, the sera precipitated a single band of M_r 210,000. The M_r 130,000 chain was heavily glycosylated in comparison to the M_r 80,000 chain. Seven of eight pemphigus foliaceus sera did not precipitate this protein or any other detectable proteins. Western blot techniques however, revealed binding of 4 of 8 pemphigus foliaceus sera to a molecule of M_r 160,000. This was the first report of a difference in the biochemical nature of the antigens that were recognized by antibodies from the two types of pemphigus.

Peterson & Wuepper (85) also reported isolation of a glycoprotein recognized by antibodies in serum from a pemphigus patient. Following urea extraction of human epidermis, the membrane fraction was extracted in SDS. A glycoprotein fraction was affinity purified on Con A-Sepharose and chromatographed on AcA 54. Binding of pemphigus antibody was detected by the Western blot technique. The pemphigus antigen was reported to be a membrane glycoprotein of M_r 66,000 composed of two apparently identical chains of M_r 33,000.

The discrepancy in the results of these two groups may be due to differing techniques. The forms with smaller molecular weight may be degradation products of the forms with larger molecular weight or the larger proteins may be precursors or aggregates of the smaller ones. It would appear, however, that a clear understanding of the biochemical characteristics of the antigen or antigens is closer than ever. As yet, attempts to make either polyclonal or monoclonal antibodies to the pemphigus antigen have been largely unfruitful; however, isolation of the antigens in an immunogenic form may increase the likelihood of raising such antibodies, which might then be used for in vitro studies of molecular pathogenecity.

ANIMAL MODELS

The lack of a good animal model system hampered pemphigus research for many years. Two recent developments in this area have been encouraging. The first of these is the recognition of pemphigus in domestic animals. The second is the development of a neonatal mouse model by Diaz and his coworkers.

Spontaneous Pemphigus in Domestic Animals

The occurrence of pemphigus in domestic animals was not documented until 1975, but pemphigus has now been reported in dogs, cats, and horses (86). The pathology and course of the disease in these animals are quite similar to those of the human disease. There are reports that some dogs with pemphigus were found to have thymomas at autopsy (87). This development opens an interesting new area of comparative dermatologic research and may provide a new approach to the study of pathogenesis.

Passive Transfer of Pemphigus Into Animals

A number of investigators have attempted to induce pemphigus in experimental animals by the passive transfer of human pemphigus sera or isolated Ig from patient sera. This approach initially brought little success. Transfusion of plasma from patients with pemphigus into monkeys resulted in intraepidermal antibody binding but no acantholytic lesions (88).

Injection of patient serum locally into rabbit or monkey skin or mucosa occasionally resulted in formation of acantholytic lesions but usually required additional chemical or physical trauma (89). More recently, athymic nude mice were grafted with human oral mucosa and injected with serum from patients with pemphigus. Although 23 of 23 injected mice showed antibody binding to grafts only 3 of 23 developed visible lesions (37).

The only in vivo model in which acantholytic lesions were reproducibly found following passive transfer of patient IgG was reported by Anhalt et al (36). They injected IgG fractions from 5 patients with pemphigus intraperitoneally into neonatal BALB/c mice (1.5–16 mg/g body weight/day). Of 55 mice, 39 developed cutaneous blisters and erosions which histologically and ultrastructurally resembled pemphigus. This report presents the strongest evidence to date that autoantibodies play a role in the pathogenesis of pemphigus. Recently, the same group has extended the ultrastructural evaluation of the mouse model system. The pathogenic events at an ultrastructural level were virtually indistinguishable from those reported for human disease (90).

THE FUTURE

Although we have made a great deal of progress in understanding the pathophysiology of pemphigus, a number of questions remains. By what mechanism does binding of pemphigus antibody to human epidermal cells stimulate the production of plasminogen activator? Our laboratory has shown that stimulation of serine proteinase activity (particularly PA) by antibody is not unique to pemphigus (91, 92). Is this a widespread activity of antibodies with specificity for cell-surface antigens? In what other disease states might this mechanism play a significant role? Will the knowledge we have gained in the studies of the molecular aspects of the pathophysiology of pemphigus provide us with a better approach to therapy than the current high-dose steroid regimen? Can specific enzyme inhibitors be employed successfully in therapy of pemphigus? Why do these patients develop autoantibodies in the first place? It is likely that efforts to answer these questions will benefit our understanding of autoimmunity in general.

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CONTENTS

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IMMUNOBIOLOGY OF MYASTHENIA GRAVIS, EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS, AND LAMBERT–EATON SYNDROME

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INTRODUCTION

The muscular weakness and excessive fatigability that characterize the disease now known as myasthenia gravis (MG) were initially described by Thomas Willis in 1672 (1). In 1973 it was shown that immunization of rabbits with acetylcholine receptors purified from fish electric organs caused similar symptoms (2). During the following decade, researchers demonstrated that the symptoms of both MG and experimental autoimmune myasthenia gravis (EAMG) are caused by an antibody-mediated autoimmune response to acetylcholine receptors (3–56). Although we now have a reasonable understanding of the complex mechanisms by which this autoimmune response impairs neuromuscular transmission, we still do not know either what initiates and regulates this response in MG or the significance of the thymoma frequently associated with it. Although muscular weakness can be partially alleviated by treatment with inhibitors of acetylcholinesterase (which increase the concentration and duration of acetylcholine to compensate for the loss of functional receptors) and although thymectomy (7, 8), immunosuppressive drugs (9, 10), and plasmapheresis (11, 12) are beneficial therapies, there is no cure for MG.

The discovery of EAMG was a by-product of the initial purification of acetylcholine receptors. Nicotinic acetylcholine receptors are now by far

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the best biochemically characterized neurotransmitter receptor (13–20). The availability of extensive libraries of monoclonal antibodies (21–37) to them and the sequencing of their subunit cDNAs (16, 17, 38, 39) should permit new levels of precision in characterizing and manipulating the autoimmune response to these receptors. EAMG has been well characterized as a model of the pathological effects of an autoimmune response to the receptor on neuromuscular transmission; the cellular mechanisms that regulate this immune response are now being scrutinized using in vitro techniques. Future studies offer the potential for integrating molecular biological studies of acetylcholine receptors with cellular immunological studies of EAMG in order to develop specific immunosuppressive therapies for MG and perhaps other autoimmune diseases.

The Lambert-Eaton syndrome (LES) (40) is a rare myasthenic condition. Researchers have recently discovered that it involves an antibody-mediated autoimmune response that impairs the release of acetylcholine at neuromuscular junctions (41, 42). The antigen has not been identified, but it may be a presynaptic calcium channel responsible for triggering the exocytosis of vesicles containing acetylcholine from the nerve ending. LES is frequently associated with small cell carcinomas of the lung, and until recently it was suspected that a factor produced by these carcinomas might be affecting neuromuscular transmission (40). Three years ago, however, investigators demonstrated that IgG from LES patients (41) could impair neuromuscular transmission in mice—like IgG from MG patients (43), though by a different mechanism. Symptomatic therapy, the use of immunosuppressive drugs, and plasmapheresis are all beneficial to LES patients (41). As with MG, the cause and cure of the autoimmune response are unknown. Furthermore, no animal model of LES comparable to EAMG exists yet, for lack of purified antigen. Whereas in the case of MG basic research on acetylcholine receptors incidentally led to the discovery of EAMG and demonstration of the autoimmune basis of MG, for LES research testing its autoimmune nature may provide antibody reagents for identifying a component of neurobiological interest. Future studies of LES should continue to parallel and contrast with studies of MG in interesting ways.

MG, EAMG (3–6, 44–47), and acetylcholine receptors (13–20, 48, 49) have been reviewed frequently and at length. Therefore, the relevant features of acetylcholine receptors and antireceptor antibodies will be reviewed quickly. Then the essential features of MG and EAMG will be discussed, concentrating on a critical review of recent developments. Finally, because of the recent discovery of its autoimmune etiology, LES will be examined briefly.

ACETYLCHOLINE RECEPTORS

Acetylcholine receptors are located at the tips of folds in the postsynaptic membrane of skeletal muscle fibers (50). Receptors are packed nearly side by side at these points, but there are only about 1×10^7 receptors per junction (51), one junction per fiber, and less than 40 μg of receptor per kilogram of muscle (52).

The function of these acetylcholine receptors is to bind acetylcholine released from the nerve ending in response to an action potential and transiently to open a cation-specific channel. This opening results in a local depolarization of the postsynaptic membrane, which can trigger an action potential which is propagated along the muscle and that triggers contraction. Two acetylcholine molecules must bind together in order to activate a receptor whose channel remains open for about 1 msec, permitting the passive flow of about 50,000 monovalent cations (53). Receptor activation is terminated both by the diffusion of the acetylcholine and by the destruction of acetylcholine by esterase located in the basal lamina between the nerve ending and the postsynaptic membrane (54). At a single fiber, neuromuscular transmission is all, if it exceeds the threshold, or none. A large safety factor is built in to ensure effective transmission. However, when the number of receptors is reduced, as in MG (51, 52), or when the number of vesicles of acetylcholine that are exocytosed from the nerve decreases, as in LES (55), transmission is likely to fail at many of the fibers in a muscle.

The structure of acetylcholine receptors from the electric organs of marine rays like *Torpedo californica* is known in great detail, and the structure of receptors from vertebrate skeletal muscle appears to be quite similar (e.g. there is 80% sequence homology between the α subunits of receptors from *Torpedo* and humans) (56). The acetylcholine binding sites are formed by its α subunits (13). There are two α subunits (M_r 50,116) and one β (M_r 53,681), one γ (M_r 56,279), and one δ (M_r 57,565) subunit in a M_r 267,757 complex (57–60). These subunits exhibit extensive sequence homology; it is hypothesized that they evolved via gene duplication and have fundamentally homologous structures (59–61). Despite these similarities, most—but not all—antisubunit antibodies are specific for a single subunit (21, 23, 26, 30, 34). All of the subunits are glycosylated (19, 58, 62), at least some have covalently bound lipids (63), and some are phosphorylated (48, 62, 64). The subunits have their N-termini on the extracellular surface of the membrane (19) and their C-termini on the cytoplasmic surface (65). The majority of the receptor is exposed on the extracellular surface and 5 transmembranous α helical domains are thought to exist in each subunit (65–67). The cation channel seems to traverse the membrane through the

center of the receptor, and the subunits are apparently oriented around this central channel. Each subunit donates a domain to form the channel, analogous to the staves of a barrel (67). Viewed from the top by electron microscopy, the receptor looks like a doughnut; viewed from the side, it looks somewhat like a funnel (68).

Acetylcholine receptors in muscle are subject to complex metabolic regulation (69). Before innervation and after denervation, receptors are found all over the surface of muscle. These receptors turn over more rapidly ($t_{1/2} < 20$ hr vs 5–7 days); open the channels longer; and differ in their isoelectric points, their pharmacological properties, and their binding of some antibodies from receptors at mature neuromuscular junctions (70, 71). These are not just two forms of receptor, "junctional" and "extrajunctional," because during development many intermediate forms are observed (70). These developmental changes probably reflect several processes of post-translational modification in addition to glycosylation (70) and phosphorylation (72), and changes in the association with lipids in the membrane, with specific components of the basal lamina (73) on the extracellular surface of the receptor, and with specific cytoskeletal components (74) on the cytoplasmic surface of the receptor.

These facts are relevant to studies of MG in several respects. Some MG patients make antibodies specific for determinants present only on extrajunctional receptors (71). This trait may reflect the origin of the immunogen in these patients. Antibodies that crosslink receptors increase the rate of endocytosis and of the lysozomal destruction of these receptors two- to threefold through antigenic modulation (75–79). This process occurs in both junctional and extrajunctional receptors (78) and must disrupt interactions with both basal lamina and cytoplasmic components. Muscles can turn over junctional receptors much more rapidly than they normally do, and the evidence suggests that at least in passively transferred MG (80, 81), this may be a compensatory mechanism for the immune assault. In MG patients, the area where the nerve ending branches becomes enlarged, perhaps in response to a budding process triggered by focal postsynaptic damage. This enlargement results in the release of larger amounts of acetylcholine, which may also act as a compensatory mechanism for the immune assault (82). Antireceptor antibodies in MG do not simply act as curare-like competitive antagonists; they also impinge on a critical and highly regulated focal point for interaction between the nervous system and muscles.

The specificities of antireceptor antibodies in EAMG have been studied using monoclonal antibodies. Most antibodies made to native detergent-solubilized receptors are directed at their extracellular surface, primarily to

the "main immunogenic region" on α subunits (21–25). The main immunogenic region has not been precisely localized on the primary sequence of α's (65, 83, 84), but it would be interesting to do so. Perhaps, like immunogenic sequences of myelin basic protein (85), analogues could function as helpers or suppressors of EAMG. Such experiments may be difficult to conduct because the main immunogenic region is partially conformation dependent. The availability of cDNAs for both *Torpedo* and human α subunits (56), however, may permit the construction of expression clones for larger domains than could be conveniently prepared by chemical methods. Most antibodies made to sodium dodecyl sulfate (SDS) denatured, purified receptor subunits are directed at the cytoplasmic surface (34, 86), and obviously would not be pathologically significant. It is possible, though, to make monoclonal antibodies to the acetylcholine binding site (27, 32, 33, 35). This rare specificity is, of course, highly effective at acutely blocking neuromuscular transmission (27).

Most antibodies to the receptor do not directly affect receptor function (87). Although antibodies to the main immunogenic region do not block function (87, 18), they can cause antigenic modulation of the receptor (88) and passively transfer the complement-dependent acute phase of EAMG (21; S. J. Tzartos, S. Hochschwender, and J. Lindstrom, unpublished manuscript). Thus, antibodies to the main immunogenic region are highly significant pathologically both because they occur most frequently and because they can cause receptor loss and postsynaptic membrane lysis. Although we do not know why this region is highly immunogenic, the existence of two α subunits on each receptor is probably important. Another potentially significant fact is that the two main immunogenic regions are oriented in such a way that an antibody (or presumably a B-cell receptor) cannot crosslink the two regions within a single receptor (88). Therefore, acetylcholine receptors should be especially effective in aggregating lymphocyte antigen receptors directed at the main immunogenic region.

Researchers have studied the specificities of antireceptor antibodies in MG patients using monoclonal antibodies of known specificities as competitive inhibitors (24). They found that the average spectrum of antireceptor antibody specificities in all patient groups is the same, and it is very similar to animals immunized with the receptor. A majority of the antibodies were directed at the main immunogenic region, but there were determinants on several subunits. Thus, MG patients do not, in general, make antireceptor antibodies with unusual specificities. In general, the response is polyclonal, so it seems that no single clone of B or T cells has run amok. An interesting exception is an MG patient with a monospecific

response to galactosamine whose antibodies appear to recognize one of the two α subunits on the receptor, bind close to its acetylcholine binding site, and recognize only a particular developmental stage of the receptor (70). This monospecific response was probably initiated differently than the polyclonal response found in most patients. The spectrum of antireceptor specificities in most patients remains constant over time, despite changes in the clinical state and antibody titer (24).

The properties of antireceptor antibodies in MG patients have also been studied using other methods. Researchers have measured the titers of antireceptor antibodies in large numbers of patients (89–93). Antibodies to the acetylcholine binding sites (which form a small part of the receptor molecule) are a minuscule proportion of the antireceptor antibodies in most MG patients (89, 91, 94, 95, 96). If they were not, these patients—or mice receiving their IgG—would succumb in hours, as do animals injected with monoclonal antibodies to the acetylcholine binding sites (27). Attempts to detect such antibodies usually test the antibody's ability to block the binding of α bungarotoxin to the receptor. Because the toxin (mw 8,000) is so much larger than acetylcholine (mw 134), the ability to block does not necessarily indicate that such antibodies bind at the acetylcholine binding site (94). Since excessive amounts of the antibody must usually be added for these blocking effects to occur, spurious inhibition of toxin binding due to microaggregate formation is a serious problem (89).

The avidity, subclass, and interspecies crossreactions of antireceptor antibodies from various patient groups have also been examined (97–108). These data provide further evidence that these antibodies are polyclonal, heterogeneous, and generally have a high degree of affinity. Patients with penicillamine-induced MG resembled patients with idiopathic MG of short duration in having a somewhat lower percentage of κ light chains, a higher percentage of IgG3, and somewhat lower avidity (98). The degree of crossreaction with receptors from other species is relatively low and varies across individuals, which emphasizes the importance of using human antigens for diagnostic assays (97, 98, 104, 106, 107). Patients with chronic ocular MG reportedly have antibodies that react better with receptors from ocular muscle than with those from other muscles (97).

MYASTHENIA GRAVIS

The pathology of MG is complex. There are probably multiple initiating events that produce the common feature of an autoimmune response to receptors, which impairs transmission through several mechanisms. Furthermore, the initiating events, the immunoregulatory events, and the

effects of the immune response are altered by hormones and trophic interactions, as well as by therapeutic intervention.

Several clinical groups of MG patients have been identified; these may reflect different modes of disease induction (91). One group (about 10–20% of the total) is characterized by thymoma, high titers of antibodies to receptors, the production of antibodies to muscle striations, and the absence of any other autoantibodies. Another group (about 20–30% of the total) is composed primarily of females less than 40 years old with intermediate levels of antibodies to receptors, few antibodies to muscle striations, an intermediate frequency of other autoantibodies, and the presence of HLA-A1, B8, and/or DR W3. A third group (about 30–40% of the total) is formed predominantly of males with lower levels of antireceptor antibodies, a high frequency of other autoantibodies, and the presence of HLA-A3, B7, and/or DR W2. A fourth, small group of MG patients have rheumatoid arthritis and were treated with D-penicillamine (98, 109, 110). Neonates from mothers with MG form the final group of MG patients. These neonates sometimes develop a transient form of MG owing to the passive transfer of antireceptor antibodies from the mother; the disease usually remits as the maternal antibodies are removed from the child (111). In addition to these forms of autoimmune MG, there are various rare congenital forms not due to autoimmune defects in neuromuscular transmission (112, 113).

There are a few tantalizing clues to the events that initiate the autoimmune response to the receptor in MG, but no solid evidence. The increased incidence of certain HLA types in some patient groups (91) suggests that in these individuals (but probably not others) some sort of immune deficiency or hyper-reactivity may be a predisposing factor. The presence of thymoma in some patients and thymic hyperplasia in many of the rest, as well as the existence of germinal centers in the thymus and the beneficial effects produced by thymectomies, certainly indicates that the thymus plays one or more important roles in most MG patients (7, 8). Thymoma is suggestive of the presence of a transforming virus. The observations that myoid cells are found in the thymus, that small amounts of receptors can be extracted from it, and that muscle cells can be grown out of thymus cultures suggest that the initial immunogen may reside there (114, 115). The fact that many antigenic determinants on the receptor are recognized in most MG patients—and recognized as in animals immunized with the receptor—suggests that the initiating immunogen is a receptor rather than a crossreacting determinant on another molecule (24). Finally, the finding that many MG patients react with determinants unique to extrajunctional receptors intimates that extrajunctional receptors are

frequently the initiating immunogen (70). But what initiates the autoimmune response? It is clear that an immune response to a receptor is not as innocuous as a response of the same magnitude to some other proteins because of the pivotal role played by receptors in neuromuscular transmission.

Pathological changes at neuromuscular junctions caused by MG include antibodies and complement bound to the postsynaptic membrane, simplification of the folded structure of that membrane, and the loss of receptors (50, 51, 116–119). Receptor loss causes loss of sensitivity to acetylcholine released from the nerve and is responsible for the weakness and fatigability that characterize MG (51, 117). Receptor loss is thought to be due to complement-mediated focal lysis (118) (which is probably primarily responsible for altering the morphology of the postsynaptic membrane) and to antigenic modulation of receptors (75–78), in which the crosslinking of receptors by the antibody increases their normal rate of endocytosis and lysozomal destruction. In addition, a small fraction of the antibodies to receptors may directly impair the latter's function; this further contributes to the loss of acetycholine sensitivity (89, 91, 94–96).

The detection of antibodies to receptors in the serum of MG patients provides an objective approach to diagnosing MG and monitoring the response to immunosuppressive therapy. Antibodies can be assayed by indirect immune precipitation using crude extracts of receptor from human muscle labeled with ^{125}Iα bungarotoxin (which binds with great specificity and affinity to the acetylcholine binding sites) as the antigen (90). In this way antibodies to receptors can be detected in about 90% of patients thought to have MG (89–93, 100). Subsaturating ^{125}Iα bungarotoxin concentrations are usually used, so that even antibodies to these binding sites can be detected. High concentrations of receptors are more easily obtained from fetal or denervated muscle. For example, using receptor from fetal bovine muscle, antibodies were detected in 80% of patients (105). Assayed under the same conditions, receptors from adult bovine muscle crossreact about 16% with human receptor (107). It is generally agreed that human receptor is the best antigen to use in these assays (89, 91, 104, 106, 107), but it is usually in short supply. An enzyme-linked immune adsorbent assay (ELISA) for antireceptor antibodies in MG patients' serums has also been developed (120). In this case a monoclonal antibody was used to anchor the receptor to the plate. This assay then obscures that determinant but permits the detection of antisite antibodies. Antibodies to receptors were detected in 80% of MG patients' serums by this method. Curiously, anti-idiotype antibodies to the mouse anti-*Torpedo* receptor antibody used to anchor receptors were detected in some of these patients (121). There have been some other reports of detection of shared idiotypes among MG patients

(122, 123), but as will be discussed in the following section on EAMG, this does not imply significant or extensive sharing of idiotypes.

The antireceptor titers in MG patients vary widely from barely detectable (e.g. 0.6 nmol of toxin-binding sites of receptor bound per liter of serum) to very high (greater than 1000 nM) with an average around 50 nM (89). Although the average titer of patients with obvious weakness only in their ocular muscles is lower than that of patients with more generalized weakness, an individual's titer does not correlate closely with the severity of his muscular weakness. Relative changes in an individual's titer are correlated with changes in the severity of the weakness, however (89–93, 124–128). The low correlation between an individual's titer and weakness probably reflects the variety of antireceptor antibody specificities present, the multiple mechanisms by which antibodies to receptors can impair neuromuscular transmission, the safety factor for transmission, and the multiple adaptive mechanisms for maintaining this safety factor. A well-known example of the difficulty of manipulating neuromuscular transmission in MG is that giving too little esterase inhibitor produces muscular weakness yet giving too much also causes weakness by overactivating the receptors, which then enter a desensitized conformation. Excessive inhibition of esterase in normal animals overactivates receptors, resulting in the excessive entry of Ca^{++} (along with other cations through the receptors), and this disrupts the postsynaptic morphology (129).

Attempts have been made to improve the correlation between measures of the amount of the antireceptor antibody and the severity of weakness by gauging the serums ability to cause antigenic modulation or to block the binding of α bungarotoxin (99, 102, 103). The difficulty with this approach is that antigenic modulation is measured on rat muscle cells (102, 103) and blockage of toxin binding is usually measured on rat (102) or chicken muscle cells (130), so that only the small and variable (80, 81. 104, 106, 107, 131) fraction of crossreacting antibodies are assayed. Developing a human muscle cell line comparable to the mouse muscle cell line BC3H-1 (76) would greatly benefit studies of MG.

Studies of lymphocyte subpopulations in MG patients have been conducted. For example, one group reported a small decrease in OKT3 and OKT8 positive peripheral blood lymphocytes (132). This group later studied thymic mononuclear cells and reported no differences in OKT3+, OKT4+, OKT6+ or OKT8+ cells (133). Another group, in contrast, reported an increase in Leu-2a and OKT8 positive suppressor cells (134). Yet another group reported a small increase in the ratio of OKT4 positive (helper) to OKT8 positive (suppressor) (135, 136) lymphocytes. Clearly, such studies of lymphocyte subsets in MG patients have not been especially informative to date.

Studies of antibody synthesis by lymphocyte cultures from MG patients have been somewhat more enlightening. The addition of *Torpedo* receptor to cultures of the peripheral blood lymphocytes of some MG patients causes thymidine incorporation (137–139). This phenomenon probably reflects the stimulation of a T-helper-cell proliferation response similar to that observed in EAMG (140, 141). Small amounts of antireceptor antibody synthesis have been detected in cultures of peripheral blood lymphocytes stimulated with pokeweed mitogen (142). Thymic lymphocytes exhibit antireceptor antibody synthesis even without mitogen (142, 143), but this unusual site of antibody synthesis is not sufficient to account for the large amount of antireceptor antibody production in these patients. A cell line producing a monoclonal antireceptor antibody has been prepared by Epstein-Barr virus transformation of thymic B cells (36). Thymic cells are known to enhance antireceptor production by peripheral blood lymphocytes, and it has been hypothesized that both antigen-presenting cells and helper T cells may contribute to this process (144). A long-term cell line of T lymphocytes reactive with acetycholine receptors from both human muscle and *Torpedo* electric organ has been prepared from an MG patient (144a). These cells display the surface phenotype of helper T cells and are genetically restricted to HLA-DR3. Their receptor-induced proliferation could be inhibited by treating the antigen-presenting cells with monoclonal antibodies to DR determinants. Future studies of long-term lines of helper, suppressor, and antibody-producing lymphocytes from MG patients could prove quite interesting.

The effects of D-penicillamine on lymphocyte cultures from MG patients have also been investigated (145). It infrequently and inconsistently stimulated both antireceptor and total IgG production. These data do not lend support to the idea that penicillamine acts by directly activating antireceptor-producing cells, but they do not exclude the possibility. Others have suggested that penicillamine may react with thiol groups on receptors and thereby provoke an immune response (146). In any case, further studies are required.

Studies of the lymphocytes of MG patients are difficult to conduct because of the small number of patients (the incidence of MG is about 1/20,000) (147), because many of the patients have been treated with immunosuppressive drugs like prednisone or azathioprine, and because of the small antireceptor response in these lymphocyte cultures. Studies of thymus cells are possible only after a thymectomy. Nonetheless, further studies at the few centers where they can be done well may help both to elucidate the events that initiate and regulate MG and to explain the beneficial effects of thymectomies.

EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

EAMG has been induced in species ranging from frogs (148) to monkeys (149), but it has been best characterized in Lewis rats (76–79, 141, 150–159) and mice (140, 160–168). Lewis rats are quite susceptible (150), whereas 100% incidence of weakness is not found even among high responder strains of mice immunized with the receptor (166). It may be that giving much higher doses of the receptor to the mice (50–100 μg) would increase the incidence of weakness. When Lewis rats are given a single injection of the receptor (15–100 μg) in complete Freund's adjuvant, and given pertussis vaccine as additional adjuvant, an acute phase of weakness is observed 8–12 days after immunization, followed by a chronic phase of weakness starting after about 30 days (150). The acute phase depends on the use of pertussis. A similar acute phase is seen within 24 hours of passively transferring antireceptor antibody to rats (154). The acute phase is characterized by a phagocytic invasion of neuromuscular junctions that occurs after the binding of antibody and the activation of complement (151, 153–156). This sort of phagocytic invasion is not observed in MG patients (6) or in mice injected with IgG from MG patients (169). The difference is probably due to the fact that the acute phase is triggered by chemotactic fragments of complement that are released at junctions in significant amounts for only short periods after the initial binding of a lot of the antireceptor antibody. Chronic EAMG closely resembles MG in that there are high titers of circulating antireceptor antibody (152, 153) and low amounts of the receptors (about 30% of normal (153), most of the receptors are bound by antibodies (153), the complement is bound to the postsynaptic membrane (158), and the membranes normal folded structure is simplified (155).

Antibodies to the main immunogenic region comprise at least half of the antireceptor antibodies made (21, 23, 25) and are pathologically significant because although they do not directly impair receptor function (18), they can cause antigenic modulation of the receptor (88) and can passively transfer EAMG (21; Tzartos et al, unpublished manuscript). The immunodominance of this small part of the receptor α subunits might suggest that only a restricted number of anti–main immunogenic region idiotypes are made. This is not the case. There is little crossreaction between anti-idiotype antibodies to one monoclonal antibody to the main immunogenic region and other monoclonal antibodies to this region (170; DeBaets et al, unpublished manuscript). Immunization of rats with monoclonal antibodies to the main immunogenic region produces anti-idiotype antibodies, but it does not diminish their response to subsequent immunization with

the receptor (170, 171). This outcome is not surprising given the anti-idiotype crossreaction results, as well as previous observations that even a simple hapten can provoke multiple idiotypes and that suppression of one of these idiotypes does not reduce the total antihapten response (172). Rabbits producing anti-idiotype antibodies to sheep antireceptor antibodies were not protected from developing EAMG either (173). These results suggest that anti-idiotype therapeutic approaches are unlikely to be effective.

Some entertaining results using anti-idiotype approaches have been obtained. Rabbits were immunized with trans-3,3′-bis[α-(trimethylammonium) methyl] azobenzene bromide (BisQ), a receptor agonist that is structurally constrained (174). Antibodies were affinity purified from these rabbits and injected into others. Some of these rabbits developed signs of EAMG. This result suggested that the binding sites of antibodies to BisQ sufficiently resembled the acetylcholine binding sites of receptors to stimulate production of anti-idiotype antibodies that inhibited the activity of receptors in muscle. In subsequent experiments (175), mice were immunized with BisQ, and hybridomas were prepared and screened for both anti-BisQ antibodies and naturally occurring anti-idiotype antibodies. Surprisingly, 14% of the wells reacted with BisQ, 7.4% reacted with rabbit anti-BisQ, and about a third of these anti-idiotype antibodies reacted with receptor. A hybridoma producing an antireceptor anti-idiotype was cloned. These experiments, like some others (176), demonstrate that antireceptor antibodies can be prepared using only ligands. They also suggest that autoimmune antireceptor diseases could originate from an anti-idiotypic response to a receptor ligand. Given the specificities of the autoantibodies actually produced by MG patients, this process is clearly not a significant initiating event in MG.

The immunogenetics of EAMG have been studied in mice (140, 160–168). One genetic component affecting the susceptibility to EAMG maps to the I-A region (140, 160–162). Mutations of the Ia molecule convert an EAMG-susceptible strain into a resistant strain (177). Monoclonal antibodies to Ia suppress the in vitro (165) immune response to the receptor of susceptible strains and reduce the response in vivo (178). The critical role of the Ia molecule may be on an antigen-presenting cell (144a) or on a suppressor cell. Another group studying the immunogenetics of EAMG has suggested that the IgC_H region may also have a significant impact by affecting the ability to produce particularly pathogenic antibody specificities (168). In murine EAMG, as in MG, the evidence suggests that no single pathological effect of an antibody (e.g. antigenic modulation, complement-mediated focal lysis, or direct antagonism of receptor function) that has been measured correlates perfectly with the severity of weakness (166–168). As in

MG, this probably reflects the existence both of multiple pathological mechanisms and of multiple compensatory mechanisms to protect the safety factor for neuromuscular transmission.

There are some interesting studies of the immune response to the receptor by lymphocytes in culture. It has been demonstrated that antigen-presenting cells are required, that after addition of the receptor T helper cells proliferate, and that the latter are required for subsequent production of antireceptor antibodies by B cells (140, 141). T-cell lines have been prepared by alternately stimulating lymphocyte cultures with the receptor (using irradiated thymocytes as antigen-presenting cells) and with a medium containing a T-cell growth factor (179). The lines have helper activity. Future studies of the properties of these lines should be fruitful. Identifying the properties of cloned cell lines specific for receptor from muscle should be especially interesting. Using a murine system, both T-helper and T-suppressor cell lines have been prepared (180). The suppressor effects were only observed when a Mishell-Dutton culture approach was used and not when the more common receptor-stimulated lymphocyte proliferation system was employed. The suppressor cells secreted an AChR-specific factor that mediated their effect. If these studies can be successfully followed up, the preparation of cloned lines producing this effect and the biochemical characterization of the secreted suppressor factor should be possible, with findings that may be relevant for specific immunosuppressive approaches to therapy.

Some approaches to specific immunosuppression of EAMG have already been mentioned. If the sequence on α subunits forming the main immunogenic region can be identified, we may find that appropriate synthetic analogues have immunoregulatory effects. Despite the large number of pathologically significant antibodies to the main immunogenic region that are made, anti-idiotype approaches do not seem promising because the complex structure of the main immunogenic region appears to provoke the formation of many noncrossreactive idiotypes (170, 171). Monoclonal antibodies to Ia may reduce the immune response to receptors (178, 144a), and investigations into variations on this approach should be pursued. If they can be prepared and characterized, specific suppressor factors secreted by cloned lymphocytes may prove to be valuable (180).

Another approach to the specific immunosuppression of EAMG is to use conjugates of the receptor with ricin toxin to kill receptor-sensitive lymphocytes specifically (181). This approach works in vitro on lymphocytes from rats with EAMG, but whether there is sufficient specificity to permit exhaustive killing of receptor-sensitive lymphocytes without intolerable damage to other tissues remains to be tested. In these experiments,

intact ricin was used that contains both a toxic subunit and a lectin subunit. In vitro studies were possible because the lectin could be blocked with high concentrations of lactose. Covalent inactivation or elimination of the lectin subunit will be required, however, before in vivo studies can be undertaken.

EAMG is not a good model of the unknown inductive events that initially provoke an immune response to receptors in MG, but it is an excellent model of the effects of the autoimmune response on neuromuscular transmission in MG. It may also prove to be a valuable model for developing a specific immunosuppressive therapy for MG. The well-characterized antigen and the complex, though not so well-characterized, pathological mechanisms are valuable assets. The rarity of MG patients compounds the difficulties of working directly with them. By taking advantage of the assets of this system, one can hope not only to learn more about acetylcholine receptors and MG but also about immunological strategies that may be helpful in understanding and controlling other autoimmune diseases.

LAMBERT–EATON SYNDROME

LES is an example of a disease that has been studied in association with MG, initially because of interesting similarities and contrasts in their symptoms and now because of recently discovered commonalities in their pathological mechanisms.

The muscular weakness characteristic of LES is caused by defective release of acetylcholine from the presynaptic membrane (40, 55, 182), instead of by defective reception of acetylcholine by the postsynaptic membrane as in MG (183). In LES the miniature end plate potentials caused by the exocytosis of quanta of acetylcholine (vesicles containing about 10^4 acetylcholine molecules) are of normal amplitude (55), whereas in MG the amplitude declines owing to the loss of receptors (52). In LES the number of quanta is reduced owing to a defective release mechanism, whereas in MG the number of quanta is normal (55). In LES there is a hypertrophy in the folded structure of the postsynaptic membrane (119)—perhaps a trophic response to the decreased release of acetylcholine—while in MG there is a decrease in the folded structure of the postsynaptic membrane owing to the immune assault (117). In some patients with either disease, there are characteristic associated cancers—small cell carcinoma of the lung for LES (40) and thymoma for MG (7). The impaired neuromuscular transmission characteristic of both LES and MG can be passively transferred to mice by chronic injections of the patient's IgG (41, 42). There are even fewer active-zone molecules than there are acetylcholine receptors, so unlike MG, bound antibody and complement have not been localized at

LES neuromuscular junctions. In LES, however, the number of large intramembranous particles characteristic of active zones in the presynaptic membrane and thought to represent voltage-sensitive calcium channels does decrease (184). Mice that have been injected with LES IgG show a similar decrease in active-zone particles (185). In both LES (41, 186) and MG (11, 12), reduction of the circulating antibody by plasmapheresis is therapeutically beneficial. LES currently lacks either the purified antigen of MG or the autoimmune model of EAMG. But the first report of purification of a potential-sensitive calcium channel (from muscle transverse tubules) has just appeared (187). This protein—or perhaps the purification of potential-sensitive calcium channels from nerve endings of electric organs or the brain maybe using LES IgG as the specific ligand—may provide the antigen to provoke an animal model of LES.

Studies of LES and MG should continue to crossfertilize one another as investigations into the induction and control of these intriguing autoimmune responses continue.

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Annual Review of Immunology
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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 133–57

FACTORS AFFECTING B-CELL GROWTH AND DIFFERENTIATION

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INTRODUCTION AND HISTORICAL OVERVIEW

Since the discovery of T-B interactions in the antibody response, the mechanism of the regulatory functions of T cells in the B-cell response has been studied extensively in immunology. The possibility that helper functions of T cells may be mediated by soluble products released from T cells was originally proposed by Dutton and his colleagues in 1971 (1). In their experiment with murine splenic B cells, they showed that anti-SRBC (sheep red blood cell) response in T cell–depleted splenic lymphocytes could be reconstituted by culture supernatants of mixed lymphocyte reactions. This was confirmed by Schimpl & Wecker (2) and several other investigators (3–5) who showed the active soluble factor(s) in culture supernatants of mitogens or antigen-stimulated T cells. Schimpl & Wecker demonstrated that cell-free supernatants from alloantigen-stimulated or concanavalin A (Con A)–stimulated T cells could fully substitute for T cells in the response in vitro to unrelated antigens such as SRBC. They postulated that T cells stimulated by their specific antigen released a substance, T cell–replacing factor (TRF), which was able to stimulate B cells to proliferate and/or differentiate only when B cells carried antigens on their receptors. Essentially the same result was reported by Kishimoto & Ishizaka with rabbit B cells (3). They demonstrated that anti-DNP (dinitrophenyl) antibody response could be induced in DNP-primed B cells by the stimulation with DNP–heterologous carrier conjugate and a cell-free supernatant from carrier–primed T cells stimulated with the primed carrier antigen. In a series of experiments, they also demonstrated that the factor(s)

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enhancing the IgE antibody response could be separated from the factor(s) involved in the IgG response and suggested that T cell–derived helper factor(s) might affect the expression of the isotype in the antibody response (6).

On the basis of the experiments done by Schimpl & Wecker (2) as well as ourselves (3), we speculated that B cells could be activated into antibody secreting cells by two signals: binding of a given antigen to immunoglobulin (Ig) receptors and T cell–derived antigen-nonspecific helper factor(s). In order to prove this notion, we attempted to induce anti-DNP antibody response in DNP–primed B cells of a rabbit with the combination of totally antigen nonspecific signals, i.e., crosslinking Ig-receptors by anti-Ig and a cell-free supernatant of antigen-stimulated T cells. In these experiments (7, 8), anti-DNP antibody response or polyclonal immunoglobulin production was stimulated by anti-Ig and T cell–derived helper factor(s). B cells stimulated with anti-Ig could absorb the activity of T cell–derived helper factor(s), and cell division was not required for the anti-Ig-dependent activation. Therefore, this study could dissect the activation process of B cells into two steps: (*a*) anti-Ig-dependent activation to the stage responsive to T cell–derived helper factor(s) and (*b*) factor-dependent proliferation and differentiation into Ig-secreting cells.

Similar attempts were made with murine B cells, but purified soluble anti-Ig reagents with some exceptions failed to activate murine lymphocytes (9). Several investigators showed, however, that anti-Ig antibody preparation, not mitogenic in soluble form, became a powerful mitogen for murine B cells when made insoluble by covalent attachment to the surface of polyacrylamide or Sephadex beads (10, 11). The effect of this might be due to the exclusion of the inhibitory signals through Fc receptors. An interesting aspect of B lymphocyte activation by anti-Ig beads was that, unlike lipopolysaccharide (LPS), they failed completely to induce the differentiation of B cells to high-rate Ig synthesis and secretion. This failure to induce the secretion of Ig was overcome by the addition of a cell-free supernatant of spleen cells activated by Con A (12), which was probably the same preparation as TRF reported by Schimpl & Wecker (2). Therefore, the result obtained in murine B cells by Parker et al (12) confirmed the previous experiments done with rabbit B cells (7, 8) and suggested that TRF substituted for the helper function of T cells in the antibody response.

In 1976, Morgan et al (13) showed that a cell-free supernatant from PHA-stimulated human peripheral lymphocytes (PBL) could induce continuous proliferation of human T cells. Based on those findings, Gillis et al (14) succeeded in the long-term propagation of murine and human cytotoxic T cells. It was soon apparent that the key element involved in the proliferation of effector T cells in culture was some moiety present in mitogen-stimulated

T-cell supernatants. In the same supernatants, TRF activity was found to induce antibody secretion in B cells; systematic biochemical fractionation of the T-cell supernatants suggested that TRF activity, thymocyte mitogenic activity, and killer helper factor activity that induces cytotoxic T cells might all be indirect measures of the same moiety, a moiety whose presence in mitogen-stimulated T-cell supernatants promotes long-term T-cell replication (15–17). Based on these results, investigators agreed that a single class of molecules was responsible for activity in a number of in vitro immune response assay systems including the antibody response and further agreed that this molecule, which could trigger T-cell replication, should be referred to as interleukin-2 (IL-2) (18). However, several experiments with more highly T-depleted B-cell populations have suggested that IL-2 alone could not substitute for the function of T cells in the antibody response (19), and extensive studies on T cell–derived lymphokines involved in the B-cell activation have been initiated. The breakthrough in this area was the discovery of B cell–specific growth and differentiation factors.

DISSECTION OF B-CELL ACTIVATION PROCESS

Previous studies (8, 12) have thus suggested that the process of differentiating B cells into Ig-secreting cells can be dissected into two distinct stages: anti-Ig-induced activation into the stage responsive to T-cell factor(s), and then T cell factor(s)–dependent proliferation and differentiation. Later, more detailed studies were done with inducible human leukemic B cells as a source of homogeneous B cells. Stimulation of leukemic B cells (B-CLL) with anti-idiotypic antibody and a cell-free supernatant of PHA (phytohemagglutinin)-stimulated T cells (PHA-Sup) induced monoclonal IgM secretion in B-CLL cells (20). Neither anti-idiotypic (anti-id) antibody nor PHA-Sup alone induced any IgM secretion, confirming the previous result that two signals are required for B-cell activation into Ig-secreting cells. Further separation of the signals for proliferation and for differentiation of activated B cells was attempted. PHA-Sup was fractionated by gel filtration on Sephadex G100 and the fraction with the molecular weight of 20 K was further separated by chromatofocusing. Anti-id antibody and the fraction focused at pH 6.5 together induced proliferation of B-CLL cells but did not induce any IgM secretion. The addition of another fraction focused at pH 5.5, together with anti-id antibody and the pI 6.5 fraction, induced proliferation as well as IgM secretion in B-CLL cells. The fraction focused at pH 5.5 alone did not induce any proliferation of anti-id antibody stimulated B-CLL cells but induced Ig-secretion in an Epstein-Barr virus–transformed human B lymphoblastoid cell line (CESS), suggesting that this

fraction was involved in the final maturation process of B cells into Ig-secreting cells. These results, therefore, clearly showed the division of the T cell factor(s)–dependent process of B-cell activation into two stages: (*a*) growth factor–dependent proliferation and (*b*) differentiation factor–dependent maturation into Ig-secreting cells. A similar result, which showed the involvement of separate signals for proliferation and differentiation of normal human B cells, was demonstrated by Falkoff et al (21). In their experiment, stimulation of human peripheral B cells with *Staphylococcus aureus* strain Cowan I (SAC) induced proliferation but no Ig-secretion. The differential response of B cells to SAC could be restored by the addition of mixed lymphocyte culture supernatants. More detailed study of the dissection of the B-cell activation process has been done by Nakanishi et al with murine B cells (22). Their result also showed the presence of three distinct steps, i.e. activation, proliferation, and differentiation. Factors involved in proliferation and differentiation of activated B cells are described in the following sections.

GROWTH FACTORS FOR B CELLS

The presence of B cell–specific growth factor(s) was originally suggested by Howard et al (23). On the basis of previous studies by Parker et al (12) and Kishimoto et al (7, 8), they employed a short-term B cell costimulator assay to measure B cell growth factor (BCGF) activity. In their study, proliferation of anti-μ-stimulated murine B cells was augmented by the addition of a culture supernatant of phorbor myristate (PMA)–stimulated EL-4 thymoma cells. Adsorption of the culture supernatant with an IL-2-dependent cytotoxic T-cell line reduced IL-2 activity from 40 u/ml to <0.1 u/ml but caused little or no reduction in B-cell costimulatory activity, suggesting the presence of B cell–specific growth factor distinct from IL-2. Gel filtration provided further evidence that IL-2 and the B cell costimulating factor are distinct entities. The major peaks of these were separable with their approximate molecular weights of 30–35 K and 16.5–18.5 K, respectively. The presence of B cell–specific growth factor distinct from IL-2 was also suggested by Pike et al (24), who used a single-cell assay method. Antigen plus conditioned media of mitogen-stimulated, cloned T-cell tumors or hybridomas triggered 5–10% of hapten-specific B cells (placed singly in culture wells) into clonal proliferation and then to differentiation into antibody forming cells. Neither antigen nor conditioned medium alone stimulated B cells into proliferation or differentiation, again showing that both signals were required for clonal proliferation of B cells. Some evidence in their study also suggested that the activity to induce proliferation of B cells and IL-2 involved distinct molecules. The factor(s) reported by Pike et

al (24) induced both clonal proliferation and differentiation of B cells, in contrast to that reported by Howard et al (23), whose BCGF induced proliferation of activated B cells but no Ig secretion. Factors with both growth and differentiation activities are discussed in the next section.

The presence of growth factor(s) specific for human B cells was also reported by several investigators. For the detection of human BCGF, either anti-Ig-stimulated B cells (20, 25), or SAC-stimulated B cells (26) were employed. By using SAC-stimulated B blast cells cultured with 0.025% SAC for 3 days, Muraguchi et al (26) demonstrated BCGF activity in the fraction with the molecular weight of 20–30 K of a culture supernatant of mitogen-stimulated peripheral mononuclear cells. They showed that BCGF activity could be separated from IL-2 activity by selective absorption of factor-containing supernatants with IL-2-dependent T cells. Thus, those experiments done with murine or human B cells suggest that BCGF and IL-2 are indeed separate molecules. Two other lines of evidence concur: (*a*) IL-2-dependent T-cell lines can absorb IL-2 activity without diminishing the activity of BCGF in the supernatants containing both factors (25, 26), and (*b*) partial separation of IL-2 and BCGF activity has been achieved by gel filtration, ion exchange chromatography, or chromatofocusing (23, 27). Recently, Farrar et al (28) highly purified murine BCGF from PMA-stimulated EL-4 cells and showed it to be distinct from IL-2 by a variety of chromatographic criteria.

The presence of BCGF distinct from IL-2 has been confirmed by the establishment of T-T hybridomas secreting BCGF molecules in mice and humans. Murine T-cell hybridomas between Con A–activated splenic T cells and the thymoma BW5147, which had been established by Lernhardt et al (29), produced B cell–specific replication factor (BRF) without any activity to induce Ig secretion in B cells (BMF), to induce proliferation of T cells (IL-2), or to stimulate myeloid or erythroid colonies (CSF). BRF from this hybridoma (A32-26) induced replication of LPS-stimulated B blasts, but no Ig secretion, showing that the factors involved in replication and maturation were distinct molecules. BRF produced by the hybridomas (A32-26) appeared in two major forms with molecular weights of 15–20 K and 80–90 K. Similar hybridomas secreting BCGF were reported by Leanderson et al (30). Their hybridomas also induced proliferation of LPS-stimulated B blasts but did not show any IL-2 activity. BCGF from their hybridomas did not support the development of Ig-secreting plaque-forming cells in B blast cultures, and terminal maturation could be induced in BCGF-dependent blasts by addition of conditioned media from normal helper T cells, again suggesting that two distinct factors were involved in T cell–dependent growth and maturation of B lymphocytes.

Human T hybridomas secreting BCGF have been established by Butler

et al (31) and Okada et al (25). Culture supernatants from several T hybrid clones induced proliferation of anti-μ-stimulated B cells or SAC-stimulated B blast cells but did not maintain the proliferation of IL-2 dependent T-cell lines. The culture supernatants did not induce any Ig secretion in SAC-stimulated B blast cells or BCDF-dependent B lymphoblastoid cell line (CESS) (25). Adsorption of the culture supernatants with anti-μ-stimulated B cells removed BCGF activity, but IL-2 dependent T cells did not reduce the BCGF activity in the supernatants (25). Moreover, the BCDF-dependent B lymphoblastoid cell line (CESS) did not adsorb the BCGF activity in the supernatants, suggesting that the expression of BCGF receptors was dependent on the activation step of B cells (25).

All of the results obtained with conventional as well as hybridoma-derived factors clearly demonstrate the presence of B cell–specific growth factor(s) (BCGF) involved in the induction of proliferation of B cells, that is (*a*) BCGFs act on B cells triggered by anti-Ig, LPS, or SAC, but not on resting B cells; (*b*) acceptors for BCGFs are expressed on activated B cells, but not on resting B cells, B cells at the final maturation stage, or activated T cells; (*c*) BCGFs are responsible for the induction of proliferation of B cells, but not for the induction of final maturation of B cells into Ig-secreting cells; (*d*) the molecular weight of BCGFs is approximately 15–20 K; and (*e*) pI value of BCGFs is 6.0–7.0. Unsolved questions with respect to BCGFs are as follows. First, BCGFs can not maintain the long-term proliferation of B cells. Thus, the question is whether BCGFs are really involved in the proliferation of B cells or are involved in the activation of B cells responsive to other growth factors not yet identified. Second is IL-2 not involved in the proliferation of B cells? And third, are there any factors with both growth and differentiation activities?

HETEROGENEITY OF BCGFS, BCGF I, AND BCGF II (BGDF)

Factor(s) With Both Growth and Differentiation Activities

The presence of two distinct kinds of BCGFs was first demonstrated by the establishment of an IL-2-dependent alloantigen-specific human T-cell clone (32). An IL-2-dependent helper–T cell clone (d4) was established from human peripheral T cells sensitized against a B lymphoblastoid cell line (CESS), and they were maintained in the presence of IL-2 together with irradiated autologous non–T cells. Upon stimulation with CESS cells, d4 cells secreted several distinct immunoregulatory molecules, and the culture supernatant showed IL-2, BCGF, and BCDF activities. By gel filtration on

Sephadex G100, IL-2 activity and activity that induces IgG in CESS cells (BCDF activity) were eluted in the fraction with the molecular weight of 15–20 K. On the other hand, BCGF activity from this particular cell line (d4) when tested with anti-id antibody stimulated B-CLL cells (33) was eluted in the fraction of 50 K. This showed a marked contrast with BCGF from PHA-stimulated normal T cells (20) or from T hybrid clones (31), which had a molecular weight of 15–20 K. The addition of 50-K BCGF from d4 cells to 20-K BCGF from PHA-stimulated T cells synergistically augmented the proliferation of anti-id antibody stimulated B-CLL cells. An interesting finding was that human 50-K BCGF could induce proliferation of murine leukemic B cells, BCL_1. Swain & Dutton reported a similar BCGF, called (DL)BCGF, secreted from a long-term alloreactive murine T-cell line, the Dennert line C.C3.11.75 (34). The activity of (DL)BCGF could be assayed on normal B cells or on the in vivo BCL_1 tumor line, and the activity was eluted in the fraction with the molecular weight of 50–70 K on gel filtration. Since 20-K BCGF reported by Howard et al (23) and several other investigators did not induce proliferation of the in vivo BCL_1 line, it would appear that (DL)BCGF or our 50-K BCGF from d4 cells were molecules distinct from 20-K BCGF.

Further studies on murine 50-K BCGF have been done by Swain et al (35), who tentatively designated 20-K BCGF as BCGF-I and 50-K BCGF as BCGF-II. BCGF-I synergized with anti-Ig in the proliferation of normal splenic B cells but had no activity when dextran sulfate was used to costimulate the same source of B cells. BCGF-I also failed to directly stimulate BCL_1 tumor B cells. In contrast, BCGF-II showed a reciprocal pattern of activity. BCGF-II failed to synergize with anti-Ig to induce proliferation of normal B cells but induced proliferation of dextran sulfate–stimulated B cells as well as the in vivo BCL_1 line (35).

Recently, we have found several Human T-cell Leukemia Virus (HTLV)-transformed cells lines that secreted a relatively large amount of BCGF-II and BCDF; these have been purified to an apparent homogeneity (Shimizu et al, in press). Human BCGF-II had the pI value of 5–6 and was eluted in the fractions corresponding to the molecular weight of more than 60-K. BCGF-II did not induce any proliferation of anti-Ig or SAC-stimulated human B cells. As observed by Swain & Dutton in murine BCGF-II (35), human BCGF-II could induce proliferation of dextran sulfate–stimulated murine B cells or nonstimulated human large B cells. Human BCGF-II could induce not only proliferation but also Ig secretion in dextran sulfate–stimulated murine B cells or in BCL_1 tumor cells. Since activities to induce proliferation and Ig secretion were copurified by several fractionation procedures, it is most likely that BCGF-II has both activities.

Similar factors with both growth and differentiation activity have been reported by several investigators. Pike et al (24), with the use of a single-cell assay for B-cell responsiveness, reported a B cell growth and differentiation factor (BGDF) secreted from EL-4 cells. Booth et al (36) showed that the myelomonocytic cell line, WEHI-3, constitutively produced a factor that stimulated the growth and differentiation of murine B cells. No physicochemical means could separate activities for growth and differentiation. Those factors with growth and differentiation activities thus may be classified as BCGF II, although the factor from WEHI-3 cells could synergize with anti-IgM for the induction of B-cell proliferation.

A factor secreted from murine T hybridomas established by Takatsu (37), designated B151 TRF, also showed both growth and differentiation activities on normal murine B cells as well as BCL_1 cells. B151 TRF induced proliferation of dextran sulfate–stimulated B cells as well as of the in vivo line of BCL_1 cells, and its activity to induce proliferation was comparable to that of (DL)BCGF (35). Furthermore, the same factor could induce IgM secretion in BCL_1 cells or in antigen-primed B cells. Therefore, those results suggest that B151 TRF may belong to the category of BCGF-II. In our recent experiment, a monoclonal antibody (B_1H_5), which was reactive with murine B cells and had the ability to inhibit human BCGF II–induced proliferation or IgM secretion in BCL_1 cells, could block B151 TRF–induced Ig induction in BCL_1 cells (Takatsu et al, personal communication). This result also suggests that B151 TRF may belong to the category of BCGF II. In polyclonal Ig secretion in anti-Ig-stimulated murine B cells, Nakanishi et al (22) demonstrated that three factors, BCGF-I, B151 TRF, and EL-4 TRF, were required for the maximum Ig induction. This result may be comparable to that observed in human B cells, in which 20-K BCGF-I, BCDF, and 50-K BCGF-II induced the maximum Ig secretion in SAC-stimulated B cells (manuscript in preparation). Therefore, B151 TRF and EL-4 TRF may belong to BCGF-II and BCDF, respectively. BCDF may be responsible for the final maturation of B cells and for the induction of high-rate transcription of mRNA specific for secretory-type heavy chains. Details about BCDF are described in the next section.

Recently, Dutton detected BCGF-II activity in a culture supernatant of PMA-stimulated EL-4 cells (38). BCGF-II secreted from EL-4 cells had an apparent molecular weight of 55-K and a pI of 5.5, exactly the same as that observed in human BCGF-II from HTLV-transformed T-cell lines. Although Dutton and his colleagues did not mention the activity of BCGF-II to induce Ig secretion, several studies described in this section demonstrated the presence of B-cell stimulatory factor(s) with growth and differentiation activity, and these may be classified as BCGF-II or BGDF.

B-CELL DIFFERENTIATION FACTORS

Factors Responsible for the Induction of High-Rate Ig Secretion

The presence of T cell-derived helper factor(s) involved in the final differentiation process of B cells into Ig-secreting cells has been suggested by Schimpl & Wecker (2, 39) and their colleagues (40). They were the first to show that helper factor(s) (TRF) were equally effective if added 48 hr after SRBC stimulation, suggesting the involvement of TRF in the final differentiation stage of B cells. Lately, they have shown that hemolytic plaque-forming cells contain radioactive label incorporated from ^{3}H-TdR only available prior to the addition of their TRF, suggesting that TRF is responsible for the induction of the final differentiation of B cells (40). Dutton also proposed that helper factor(s) brought about the final differentiation of the B cells, in which clonal expansion had already been initiated by exposure to antigen alone (41).

The presence of B cell differentiation factor(s) (BCDF), which does not have growth activity and is involved in the final differentiation to high-rate Ig secretion, is demonstrated by employing a monoclonal human B-cell line as target cells. Muraguchi et al (42) established a human B lymphoblastoid cell line (CESS) that was responsive to BCDF. IgG secretion was induced in CESS cells within 48 hr by the addition of helper factors from PHA-stimulated T cells. BCDF did not affect the proliferation of CESS cells, and the blocking of cell proliferation with hydroxyurea did not inhibit the increase of IgG-secreting cells. Absorption of PHA-sup with CESS cells removed BCDF activity but not IL-2 activity. The result suggested that CESS cells expressed receptors for BCDF but not for IL-2, and IL-2 was not involved in the induction of IgG in CESS cells. No involvement of IL-2 in CESS induction was confirmed by recombinant IL-2, which will be described in the next section. Employing Northern blot analysis, we have recently shown that BCDF induces an increase in biosynthesis and secretion of γ-chains in CESS and that BCDF induces a preferential increase in mRNA specific for secretory γ-chains (Kikutani et al, in press). Saiki et al (43) established another B lymphoblastoid cell line (SKW 6 CL-4), where BCDF induced IgM secretion, and Kikutani et al also demonstrated (43) a BCDF-induced increase in mRNA for secretory μ-chains. These results with monoclonal cell lines clearly showed the presence of the factor(s), BCDF, involved in the final maturation of B cells to high-rate IgG and IgM secretion.

The activity to induce IgG or IgM secretion in B cell lines was separated from the activity to induce proliferation of anti-Ig-stimulated B cells by

chromatofocusing (20). BCDF activity released from PHA-stimulated human T cells had a molecular weight of 20-K and a pI of 5.5–5.7. Teranishi et al reported a similar result with BCDF-inducible B cell lines (44), where BCDF was found in 20-K as well as 35-K fractions. BCDF activity to induce IgG in CESS cells was detected in the culture supernatant of a human T hybrid clone, 90-E, established by Okada et al (25). This supernatant did not have any activity to induce proliferation of anti-μ-stimulated B cells or an IL-2-dependent T-cell line, confirming the presence of the factor only in the final differentiation of B cells. Recently, Butler et al (45) also reported the establishment of a human T hybridoma secreting BCDF. In their study, BCDF from a hybrid clone induced Ig secretion in SAC-stimulated B cells but did not have any activity to induce proliferation of B or T cells. BCDF activity was eluted in the fraction with the molecular weight of 30–35 K and with a pI of 5.9. Their BCDF activity was also absorbed with CESS cells. Human BCDFs secreted from PHA-stimulated T cells, T hybridomas, or HTLV-transformed T-cell lines (Shimizu et al, in press) have the molecular weight of either 20-K or 30–35 K and a pI of approximately 5.5.

Murine BCDF activity was also detected by BCDF-reactive B-cell lines. Pure et al (46) employed a murine leukemic B cell line, BCL_1, and demonstrated IgM induction in those cells by incubation with supernatants from T-cell lines, clones or hybridomas. The activity to induce IgM secretion in BCL_1 cells or splenic B blast cells was designated as BCDFμ (47). When BCL_1 cells were cultured with BCDFμ, they secreted IgM at levels which were 10–90 fold above those found in the media of cells cultured without BCDFμ, and Northern blot analysis showed a striking increase in the quantity of mRNA encoding the secreted form of the μ chain (48). BCDFμ did not have IL-2, γ-IFN or BCGF activity and a molecular weight was between 30–60 K. Another murine B cell line, WEHI-279, or pre–B cell line, 70Z/3, were also employed for the detection of BCDF activity. By incubation with culture supernatants from cloned T cells including BCDF activity, WEHI-279 cells shifted the ratio of μ chains produced from mostly membrane to mostly secretory type and begin to secrete large amounts of IgM (49). In 70Z/3 cells, BCDF induced the synthesis of κ chains and the expression of membrane IgM that was detectable by immunofluorescence (49). 70Z/3 cells and WEHI-279 cells, however, were responsive not only to BCDF but also to IL-1 (50) or γ-IFN (51) and those factors could induce IgM expression on 70Z/3 cells. Therefore, 70Z/3 cells may not be a suitable target for the detection of BCDF.

By employing WEHI-279 or 70Z/3 cells, Sidman et al (52) have highly purified murine BCDF from a culture supernatant of a helper T-cell line.

The molecules active in the induction of WEHI-279 appeared distinct from several other lymphokines, IL-1, IL-2, G/M-CSF, IFN, IL-3 and BCGF. The molecules were mildly acidic, pI 5–6 and with an apparent molecular weight of 50–55 K by gel filtration and 16-K by SDS-PAGE. They designated this factor as B cell maturation factor (BMF). The interesting point was that BMF could directly activate normal resting B cells into Ig-secreting cells (53). A factor which stimulates resting B cells to mature to Ig secretion (BRMF) has also been reported by Melchers et al (54). BRMF or BMF appeared to cause polyclonal Ig secretion by all subsets of normal B cells examined including neonatal B cells, B cells from various organs, nu/nu B cells and B cells from CBA/N mice (53). If BMF or BRMF could act on normal resting B cells, they might be responsible for the polyclonal activation of B cells in autoimmune diseases or clonal abortion of immature autoreactive B cells. Actually, proliferating T cells in enlarged nodes and spleens of older MRL/1 mice, in the absence of mitogens, secrete in vitro abnormally high levels of a factor (L-BCDF) inducing terminal differentiation of B cells into Ig-secreting cells (55). As described, a factor from WEHI-3 cells (36) or B151 TRF (37), which showed both growth and differentiation activities, could also act on normal resting B cells. Therefore, those factors with differentiation or both growth and differentiation activities might be involved in the pathological activation of B cells.

Hirano et al (56) demonstrated that human BCDFs, which induce Ig secretion in B lymphoblastoid cell lines, do not induce the maximum Ig secretion in SAC-stimulated normal B cells. Another factor (or factors) tentatively designated as BCDF-I, which has an apparent molecular weight of 20-K and a pI 6.5–7.0, was required for the maximum induction. The need for another factor besides BCDF for the maximum activation of B cells into high-rate Ig secretion has been shown by several investigators (57–59). Swain et al (57) demonstrated that the preparation included IL-2 activity and culture supernatants from a longterm alloreactive T-cell line, which were relatively inactive if added alone in anti-SRBC response of T-depleted B-cell population but showed a marked synergy when added together. Leibson et al (58) also showed an essentially the same result. Both IL-2 and another factor with 40 K mol.wt. were required for anti-SRBC response in a T-depleted B cell population. Parker's experiment (59) also demonstrated that the factor that enabled activated B cells to mature to high-rate Ig secretion required the presence of another factor including IL-2 for the maximum Ig induction in activated B cells. Elkins and Cambier (60) established that BCDF secreted from a murine–T hybrid clone was also optimally effective in the presence of small amounts of EL-4 supernatant. Therefore, all of those studies have shown that certain factor(s), possibly IL-2, synergize with BCDF for the maximum Ig induction in activated B cells.

However, the active moiety of BCDF-I fraction described by Hirano et al may not be IL-2. Ralph et al (61) reported a B-cell inducing factor (BIF), which could induce a high number of Ig-secreting cells in SAC-stimulated B cells. This factor may be comparable to Hirano's BCDF-I and their results suggest the presence of two distinct kinds of human BCDFs.

As described, not only T cells, but also a monocytic tumor cell line, WEHI-3, could secrete BCDF-like activity. Yoshizaki et al (62) showed that a human B cell line secreted B cell line-derived BCDF (B-BCDF) and this could induce IgG and IgM secretion in CESS and SKW6 CL-4 cells, respectively. B-BCDF also induced Ig secretion in SAC-stimulated B cells when another fraction with a pI of 6.5 to 7.0 (BCDF-I) was present. B-BCDF has been highly purified to an apparent homogeneity by employing FPLC (Fast protein liquid chromatography, Pharmacia) and HPLC (High performance liquid chromatography, Waters). B-BCDF had a molecular weight of 20-K, a pI of 5.1 and eluted between 45–47% of acetonitrile by reverse phase column chromatography. The result strongly suggests that even B cells are able to secrete BCDF with physicochemical and immunological properties similar to T cell–derived BCDF. It may explain the reason why T-independent antigens or mitogens are able to induce Ig secretion in T depleted–B-cell population.

ISOTYPE REGULATION BY BCDF

There has been increasing evidence of the role of T cells in isotype specific responses in all Ig classes. Kishimoto & Ishizaka (63) originally suggested the presence of isotype-specific regulatory T cells in the IgE antibody response. In anti-hapten IgG and IgE responses in vitro, they demonstrated that carrier primed–T cell populations showed helper effect preferentially either on the IgE or on the IgG antibody response. They also demonstrated that helper factors for the IgE and the IgG antibody responses were physicochemically separable and proposed that distinct molecules were involved in the differentiation of Bε and Bγ cells into IgE and IgG secreting cells, respectively (6). On the basis of those results, we have succeeded in the induction of murine IgE class-specific suppressor T cells, which showed the suppressive effect selectively on the IgE antibody response (64). In a series of experiments (65), IgE class-specific suppressor T cells were shown to secrete IgE-binding suppressor factor(s) which are able to bind with IgE molecules on the surface of Bε cells and inhibit their differentiation or biosynthesis and secretion of IgE molecules (66). Ishizaka and his colleagues (67–70) observed exactly the same result in the IgE response of rats. Very recently, they cloned the gene for the IgE binding factor from a rat T hybridoma

secreting IgE-binding suppressor factor (71) (K. Ishizaka, personal communication).

With respect to the IgA antibody response, a similar isotype-specific regulatory mechanism of T cells has been proposed. Kiyono et al (72) established SRBC-specific helper T-cell clones from murine Peyer's patch T-cell population primed with SRBC. Those clones expressed Fc receptors for IgA and showed helper effect preferentially on anti-SRBC antibody response of IgA class. When B cells were separated into $sIgA^+$ and $sIgA^-$ populations, those helper T-cell clones showed IgA-specific helper effect only on the $sIgA^+$ B cells population. Their results suggested that IgA binding factor(s) from helper T cells were involved in the differentiation of $sIgA^+$ B cells into IgA secreting cells, as demonstrated in the IgE antibody response. Mayer et al (73) demonstrated the secretion of IgA specific helper factor(s) from a human T-hybrid clone. The culture supernatant from a hybrid clone induced IgA–plaque forming cells (PFC) when added to tonsillar or peripheral blood non-T cells. Complete differentiation into IgA plasma cells was observed not only in normal B cells but also in B-CLL cells bearing IgA on their surface. Depletion of $sIgA^+$ B cells abrogated the effect of IgA specific helper factor(s) and did not induce the IgA response. This provides evidence that IgA specific helper factor does not appear to be effecting an Ig class switch, but acts on post-switch IgA committed B cells. On the other hand, Kawanishi et al (74) suggested the presence of IgA-specific switching T cells. They have cloned helper T cells from Con A–activated Peyer's patch T-cell population. Those cloned T cells induced an preferential increase of $sIgA^+$ B cells but no IgA secreting cells when cultured with $sIgM^+$ B cell population, indicating that those T cells induced switching of $sIgM^+$ B cells into $sIgA^+$ B cells.

Extensive studies of BCDF-regulating IgG_1 antibody response have been done by Vitetta and her colleagues (75). Stimulation of murine splenic B cells with LPS resulted in secretion of IgG_3. The addition of culture supernatants derived from T cell lines (PK 7.1 or EL-4) or a T hybridoma (FS 7) with LPS resulted in a pronounced increase in secretion of IgG_1. The factor which induced IgG_1 secretion was designated as BCDFγ. BCDFγ had a molecular weight of 15–20 K, suggesting that it was different from BCDFμ, which induced IgM secretion in BCL_1 cells or normal splenic B cells. The activity of BCDFγ was not absorbed with Sepharose bound to any class of immunoglobulins; this showed a marked contrast to IgE-binding helper or suppressor factors (65–71). The receptor for BCDFγ was thus apparently not sIg and this was confirmed by the response of $sIgG^-$ cells to BCDFγ (47). These results indicated that BCDFγ was not involved in the selective expansion or in the induction of differentiation of $sIgG_1$

positive B cells, but was involved either in the switch induction of precommitted cells into γ_1-secreting cells or in the induction of post-committed but sIgG$^-$-B cells.

All these experimental results clearly show that certain T cell–derived molecules are involved in the regulation of the isotype expression in the antibody response. The factors regulating the isotype expression could be divided into two groups: (*a*) the factors which have Ig-binding activity and are involved in the selective expansion or differentiation of committed B cells; and (*b*) the factors which do not have Ig-binding activity and might be responsible for the switch-induction.

IL-2 AND γ-IFN AS B-CELL STIMULATORY FACTORS

The involvement of B cell–specific growth and differentiation factors in the activation of B cells to Ig secreting cells has been clearly demonstrated. However, the role of IL-2 or γ-IFN in the activation of B cells, that is, whether IL-2 or γ-IFN has any direct effect on proliferation or differentiation of B cells, has not yet been solved. In a series of experiments done by Kappler and his colleagues (58, 76–78), they have suggested the involvement of IL-2 and γ-IFN in the induction of anti-SRBC response of murine B cells. They demonstrated that three helper factors showed synergy in the plaque response of B cells to the antigen SRBC. The first was IL-2 containing supernatant of T hybridomas (FS6); the second was factor(s) with the molecular weight of 30–50 K without IL-2 activity, and the third was an IL-1 containing supernatant from a macrophage cell line, P388D$_1$. In the subsequent experiment, Kapler et al suggested that the second factor might be γ-IFN. Similar results were reported by Swain et al (57) and Parker et al (59). As already described, IL-2 containing supernatants showed a marked synergy with culture supernatants which included the activity of BCDF. All of those results suggest the direct effect of IL-2 on B cell activation. In contrast, several studies state that it is unlikely that IL-2 directly affects B cells, i.e. (*a*) activated B cells do not absorb IL-2 activity in the murine and human system (25, 26); (*b*) highly purified IL-2 from EL-4 cell line has little effect on B cell proliferation in the absence or presence of polyclonal B cell activators (23, 28) and IL-2 activity can be separated by biochemical procedures from BCGF and BCDF in the murine as well as human system (23, 27, 28), and (*c*) IL-2 receptors or IL-2 binding can not be detected on activated murine B cells (79, 80).

In order to prove or disprove the direct effect of IL-2 on B cells, IL-2 preparation should not include any other lymphokine activities and B-cell

preparation should not include any T cells which might be activated to secrete B-cell stimulatory factors in the presence of IL-2 (81). Cloning of a cDNA for IL-2 by Taniguchi et al (82) has made it possible to obtain theoretically pure IL-2 as transcription products of a cloned cDNA. Our recent study with recombinant IL-2 has shown that IL-2 is able to induce proliferation of SAC- or anti-μ-activated human B cells (Nakagawa et al, in press). Of activated B cells, 20–30% expressed Tac, an epitope found on the human T cell IL-2 receptor, and anti-Tac antibody completely inhibited IL-2 induced proliferation of activated B cells. That IL-2 activated contaminating T cells to secrete B cell stimulatory factors is very unlikely, since no Leu 1- or Leu 4-positive cells were detected in highly purified B blast populations. Furthermore, stimulation of T cells with anti-μ and IL-2 did not induce a significant proliferation of T cells, suggesting that anti-μ did not activate T cells into IL-2 reactive stage. However, this result does not necessarily mean that IL-2 is essential for the growth of B cells. In our study, 10–100 units/ml of IL-2 could induce the maximum proliferation of activated B cells. On the other hand, conventional T cell factors including 0.1 unit/ml of IL-2 could induce the maximum response which was higher than that observed with 10 units of IL-2. The result suggests the essential role of B cell–specific growth factor(s) in the proliferation of B cells, although a relatively high concentration of IL-2 is able to induce proliferation of B cells.

Similar results have been observed by Tsudo et al (83) and Zubler et al (84) in the human and murine systems, respectively. Tsudo et al (83) demonstrated the expression of Tac antigen on SAC-stimulated B cells and IL-2–induced proliferation of those B cells. Zubler et al (84) observed that murine B cells which have been maximally activated with anti-Ig plus LPS, expressed about 3500 IL-2 receptors per cell and IL-2 could induce optimal B-cell proliferation. Tac expression on human malignant B cells or B-cell lines has also been reported by several other investigators. Korsmeyer et al (85) originally reported that Tac antigen was found on hairy cell leukemic cells that also expressed B-cell surface markers. They have shown rearrangement and expression of Ig genes in these malignant cells. Longo et al (86) demonstrated the expression of Tac antigen on HTLV-transformed B-cell lines. No EBV-transformed B cell lines expressed Tac antigen, but all HTLV-transformed T- and B-cell lines expressed Tac antigens. Therefore, HTLV might have an effect on the expression of Tac antigen. All of those results indicate that B cells are able to express IL-2 receptors in certain conditions and IL-2 can induce proliferation of those B cells.

In our study with recombinant IL-2 and γ-IFN, IL-2 induced proliferation of SAC-stimulated B cells but no Ig secretion. The addition of γ-IFN

with IL-2 induced Ig secretion comparable to that with conventional T-cell factors (Nakagawa et al, in press). Similar results with murine B cells have recently been reported by Leibson et al (87) and Sidman et al (51). Thus, IL-2 appears to be one of the growth factors for B cells and γ-IFN one of the differentiation factors for B cells. In a certain condition, however, IL-2 may induce differentiation of B cells into Ig-secreting cells. In our very recent experiment B hybridomas, which had been established by fusion of SAC-stimulated B cells with murine B lymphoma (M12) (88), were reactive to BCDF as well as to IL-2 (Kishi et al, submitted). IgM secretion was induced in those hybrid cells either with BCDF or with IL-2 and inhibited by anti-Tac antibody with IL-2 but not BCDF. However, IL-2 did not affect the growth of these hybrid cells. Ig secretion increased by IL-2 was also observed in activated murine (Nakanishi, personal communication) or human B cells (Muraguchi, personal communication). It may be reasonable to assume that IL-2 can act on any cells expressing IL-2 receptors, and the effect of IL-2 on the growth or differentiation may depend on the activation stage of target cells.

FACTORS INVOLVED IN THE ACTIVATION OF RESTING B CELLS

In the preceding sections, I have described factors affecting the growth and differentiation of B cells and demonstrated that 3 signals—crosslinkage of Ig receptors with anti-IgM or multivalent antigens; B cell specific growth factors (BCGFs); and B cell specific differentiation factors (BCDFs)—are required for the induction of proliferation and Ig secretion in B cells. This principle, however, may not be applied to the activation of a certain subpopulation of B cells or to B cells at the complete resting stage.

Several studies in the murine system have demonstrated that the activation signals required to trigger antibody production by B cells vary considerably depending on the nature of the B-cell subpopulation under study. Andersson et al (89) separated B cells by cell size or density into activated blast cells and small resting B cells and demonstrated that T cell–derived helper factors could induce polyclonal Ig secretion in B blast cells, whereas H-2 restricted T-B interactions were required for the activation of small resting B cells. Several investigators have also demonstrated the requirement of direct T-B interactions for the activation of resting B cells by employing helper T cells specific for B cell–surface determinants, including male associated (HY) antigen (90–92), allogeneic Ia antigens (89), minor histocompatibility determinants (93) or covalently bound hapten (94). Recently, Noelle et al (95) showed that TNP antigens with T cell–derived growth and differentiation factors did not induce proliferation or differenti-

ation of highly enriched TNP-binding B cells; linked recognition of TNP antigens with carrier-primed T cells was required for the activation of those TNP-specific B cells. Principate et al (96) also demonstrated, using TNP-specific human helper T-cell clones, that direct contact between T cells and TNP-modified autologous small B cells was required for the activation of small B cells. In a series of experiments with $Lyb5^-$ and $Lyb5^+$ B cells, Asano et al (97, 98) clearly demonstrated that the activation of hapten-specific $Lyb5^-$ B cells requires both hapten-carrier linkage and H-2-restricted T-B interactions. On the other hand, the triggering of hapten-specific $Lyb5^+$ B cells is genetically unrestricted and does not require carrier-hapten linkage.

Then, the questions are (*a*) What kind of signals are generated by direct interactions between H 2–restricted T and B cells; (*b*) What is the relationship between factor-mediated activation and the activation by direct interactions of T and B cells? Xid B cells from CBA/N mice or NBF_1 male mice do not respond to BCGF and/or BCDF for proliferation and Ig secretion. However, when Xid B cells were cocultured with an H 2–restricted helper T-cell clone for 24 hr, proliferation and Ig secretion could be induced in those cells with BCGF and BCDF, showing that direct interaction with a helper T cell clone activates Xid B cells to the stage responsive to BCGF and/or BCDF (99). In order to study the H-2 restriction in polyclonal activation of Xid B cells, KLH-specific helper T-cell lines from NBF_1 ($H\text{-}2^{k/d}$) male mice were cloned and 2 clones which showed a different H-2 restriction pattern were selected. A clone, MK6, was activated by antigen presenting cells with $H\text{-}2^d$ phenotype even in the absence of KLH, while another clone, CK4 showed a restriction to $H\text{-}2^k$ determinant and was activated by KLH plus accessory cells with $H\text{-}2^k$ phenotype. Coculture of NBF_1 male B cells either with MK6 or with CK4 could induce Ig secretion. However, B cells from CBA/N mice ($H\text{-}2^k$) were activated only with CK4 cells which were restricted to $H\text{-}2^k$ determinants. Even in the presence of accessory cells with $H\text{-}2^{d/k}$ phenotype, MK6 clone did not activate B cells from CBA/N mice. Moreover, the addition of monoclonal antibody, 17/227, which was specific for $I\text{-}A^k$-determinant, could inhibit Ig-induction in NBF_1 B cells by $H\text{-}2^k$ restricted CK4 clone, but not in the same B cell population by $H\text{-}2^d$ restricted MK6 clone (99). All these results suggest that the signal provided by direct interactions of H-2-restricted T and B cells may be generated by recognition of Ia molecules on the surface of target B cells by Ia-restricted T cells and that the signals may be transduced through Ia molecules on B cells. Mature B cells express Ia molecules on their surface and many B lymphomas possess antigen-presenting cell (APC) activity to T cells (100, 101). A recent study has shown that even resting small B cells can act as APC for T cell activation (102).

However, anti-Ig stimulation or some lymphokines, i.e. γ-IFN or IL-1, can induce hyper-Ia expression (103–106) and such activated B cells are more effective for the activation of T cells. Therefore, in the H-2-restricted T and B cell interactions, T cells may be activated by B cells and then activated T cells may provide signals through Ia molecules for the activation of B cells. Some lymphokines from activated T cells as well as the antigen binding to Ig receptors induce an increased expression of Ia molecules on B cells, which exert a positive feedback effect on the H-2-restricted activation of T and B cells by T-B interactions.

Then, the question is whether the H-2-restricted activation of resting B cells by T cells can be replaced by certain soluble factors from T cells. A recent study done by DeFranco et al (107) has suggested the involvement of non H-2-restricted factors in the activation of resting B cells or Xid B cells by the H-2-restricted T-B interactions. In their study, B cells that do not bear Ia molecules for which the T cells are cospecific can be activated if antigen and a source of antigen-presenting B cells of proper histocompatibility type are present. The presence of non-H-2-restricted factors may be responsible for the induction of receptors for B-cell stimulatory factors on resting B cells. Some BCDFs or BCGFs which can act on resting B cells might be considered as factors responsible for the induction of BSF receptors on resting B cells.

NOMENCLATURE FOR B-CELL FACTORS

As has been described, a number of factors involved in B cell activation have been reported and this field is currently hampered by competing nomenclature. It is evident that there may be more distinct names than factors. However, no factors have yet been isolated and it is not known how many distinct factors are really involved in B cell activation. In such a situation, a group of scientists interested in the lymphokines that regulate the activation, growth and differentiation of B cells met in Kyoto, Japan, in August, 1983, to consider the nomenclature of these substances. The group proposed that factors that had been well characterized functionally and chemically preferably by more than one laboratory, be given a formal designation. Such a designation would be of the form: B-cell stimulatory factor (BSF), followed by a consecutive number (i.e. $1, 2, \ldots, n$). Unless the factor had been purified to homogeneity or its structure determined from cloning and sequencing of its gene, a "P" for provisional would precede this number. As a result of reviewing available data, the group concluded that 20-K BCGF in mice and humans had been sufficiently well characterized to warrant a BSF designation and was designated BSF-PI. The working group also suggested a formula for the naming of newly described factors

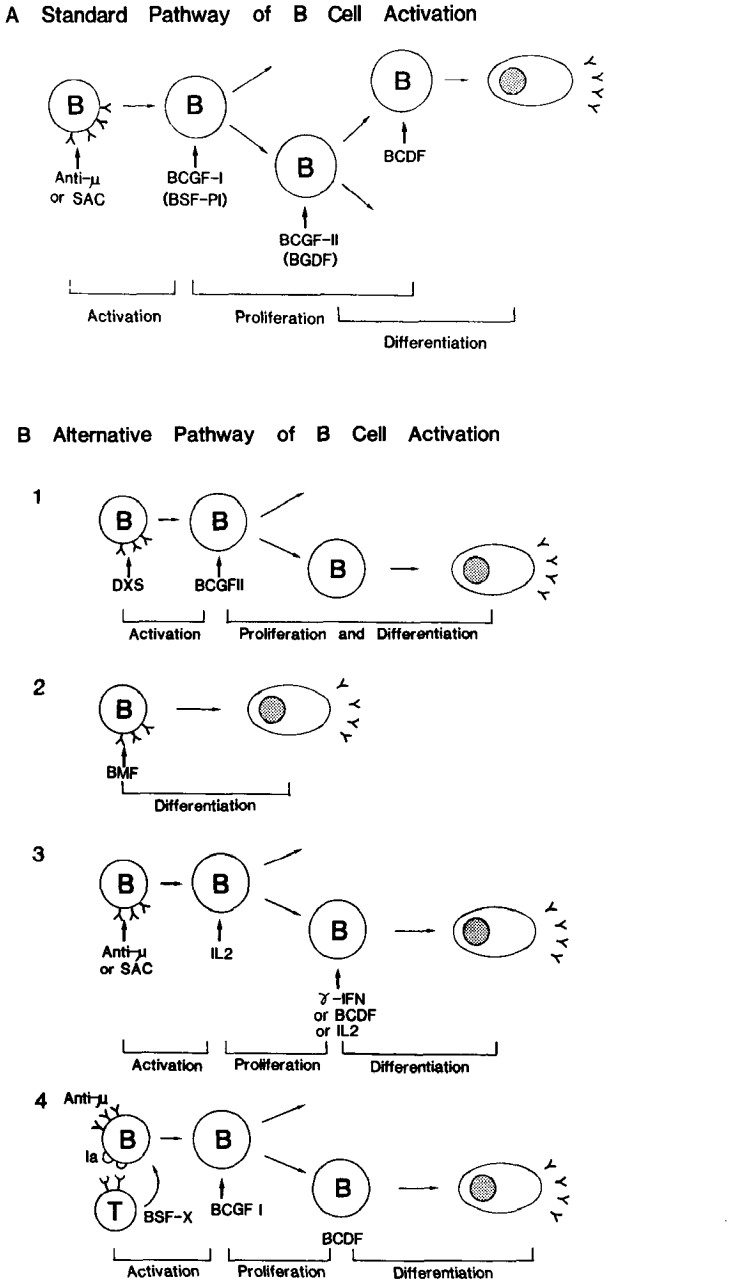

Figure 1 Schematic depiction of the activation process of B cells and signals required for the process.

before they receive a BSF designation. Such names would have three parts: first, an abbreviation of the cell source of the factor (e.g. d4 for human helper T-cell line, d4); second, an abbreviation of the activity of the factor (e.g. BCGF for B-cell growth factor); and third, an abbreviation of the name of one of the authors describing the factor (e.g. yos for Yoshizaki). Thus, 50 K BCGF would be termed d4-BCGF-yos. As this nomenclature is not yet familiar, I employed in this review an old terminology, which is customary for the scientists in this field, such as BCGF-I, BCGF-II (BGDF), and BCDF.

CONCLUSION

It is now clear that factors which are T cell-derived, antigen non-specific and genetically non-restricted are involved in the activation of B cells into antibody secreting cells. Many B-cell stimulatory factors with distinct functions have been reported in mice and humans. At present, no factors have yet been isolated and it is not known how many factors are really involved in the activation of B cells. However, as has been described, they can be divided into three groups, (i) factors with the activity to induce the growth of activated B cells; (ii) factors with the activity to induce the final differentiation of B cells into Ig-secreting cells and (iii) factors with both growth and differentiation activities. A single factor, BCGF-I or BSF-PI with the molecular weight of 15–20 K, would be responsible for the induction of proliferation of anti-μ activated B cells. On the other hand, many factors with differentiation activity have been reported and it is not known whether several distinct BCDFs are required for Ig-secretion in a single B cell or whether different B-cell subsets require BCDFs with distinct functions. The activation process of B cells and the signals required for each step of activation are summarized in Figure 1. The isolation of several B-cell stimulatory factors and the cloning and sequencing of their genes should be accomplished in the near future and the activation mechanisms of B cells may be revealed at molecular level by employing theoretically pure recombinant BSFs.

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CONTENTS

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MECHANISMS THAT REGULATE IMMUNOGLOBULIN GENE EXPRESSION

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INTRODUCTION

The expression of genes encoding the light- and heavy-chain immunoglobulin polypeptides is stringently regulated: (*a*) the genes are expressed only in cells of the B-lymphocyte lineage; (*b*) heavy-chain genes are expressed earlier in B-cell development than light-chain genes; (*c*) after light-chain genes are activated, expression of the two chains is coordinately regulated; (*d*) levels of immunoglobulin gene products increase by several orders of magnitude as pre-B cells mature to terminally differentiated plasma cells; and (*e*) immunoglobulin molecules may be either displayed on the cell surface of B cells or secreted or both (1). Furthermore, the growth and maturation of B lymphocytes, with its concomitant changes in immunoglobulin gene expression, are modulated by a variety of signals received at the B-cell surface from antigen, T cells, or T-cell factors (2). Since their organization and structure are well-established (3), immunoglobulin genes provide an excellent opportunity to unravel a complex regulatory circuit and to understand the molecular bases for the exquisite sensitivity and control of the vertebrate immune system.

Immunoglobulin genes also display several characteristics that appear to be unusual for eukaryotic genes; (*a*) DNA rearrangement is required for their expression (3); (*b*) only one of the two chromosomes is functionally expressed, a phenomenon called *allelic exclusion* (4); and (*c*) they appear to be members not only of three multigene families (5) but also of a "supergene family" which includes genes of the major histocompatibility complex, the T-cell receptor genes, and genes for other proteins found on the cell surface

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of lymphoid cells. However, other systems in higher organisms such as sensory or neural systems probably express information that is as diverse and exquisitely regulated as that of the immune system. When more is learned about the genes encoding such complex systems, they may be found to share some of the "unique" properties of immunoglobulin in genes.

This review focuses on work that elucidates the molecular events involved in regulating the synthesis, processing, utilization, and stability of immunoglobulin RNA and protein. This area was reviewed by Wall & Kuehl in 1983 (1) so that only studies since that review will be considered in detail. The rapid progress in the field is evident and an even greater explosion in our knowledge of immunoglobulin gene regulation is likely in the near future.

CHROMATIN STRUCTURE OF IMMUNOGLOBULIN GENES

Chromatin Structure and Active Genes

DNA in the nucleus of eukaryotes is wound around histone cores, bound by nonhistone proteins, and compacted into highly ordered chromatin structures (6). Differences in the chromatin structure of a particular gene could potentially have profound effects on its regulation: Different DNA-protein interactions might allow regulatory molecules and enzymes differential access to specific regions of the gene; different folding of the DNA might bring sequences that are distant in the linear duplex into close proximity with one another; and compaction of DNA into chromatin might introduce differential amounts of superhelicity into particular DNA regions. Due to its complexity, native chromatin structure is difficult to study. However, certain identifiable characteristics of chromatin structure are clearly associated with actively transcribed genes.

Increased sensitivity to digestion with DNase I or a variety of other nucleases has consistently correlated with actively transcribed regions (6, 7). The extent of the region sensitive to nuclease digestion often extends several kilobases on either side of the gene and may represent a generally more open or accessible structure of the DNA and/or the presence of different nonhistone proteins in this region (8). Sensitivity to nuclease digestion precedes expression of some developmentally regulated genes, suggesting that it may be involved in the poorly understood process of commitment of a cell to expression of a particular gene (9, 10).

In addition to general nuclease sensitivity, active genes usually have specific, short regions that are hypersensitive to DNase I digestion (11). The functional significance of hypersensitive sites is not completely understood,

but we know they can occur at nucleosome-free regions (12, 13), at promoters, at regions with the potential to form Z DNA, at enhancer regions, and at transcriptional termination sites (11). The common feature of such sites may be specific binding of nonhistone protein. In some cases hypersensitive sites have a unique primary structure which forms single-stranded regions detectable with S1 nuclease (14).

The extent to which particular CpG pairs are methylated, although not strictly a characteristic of chromatin structure, is another feature of DNA correlated with gene activity in many cases. Here it is discussed under "chromatin structure." Actively transcribed genes are often hypomethylated and nontranscribed genes are often heavily methylated (15, 16). Since methylation of a single CpG pair can be sufficient to alter expression of a gene (17), studies on general methylation patterns can be misleading or difficult to interpret. Changes in DNA methylation require one or more rounds of replication; thus methylation is probably used for long term forms of gene regulation.

Chromatin Structure of Immunoglobulin Genes is Different in Lymphoid and Nonlymphoid Tissues, and Changes During B-Cell Development

SENSITIVITY TO NUCLEASE DIGESTION AND METHYLATION Immunoglobulin genes in nonlymphoid tissues such as liver or kidney are insensitive to digestion with DNase I (18–22) and heavily methylated (18, 23, 24), consistent with the fact that they are not expressed in these tissues. However, distinct changes are observed in lymphoid cells.

C_μ constant region gene segments in pre-B cells are sensitive to DNase I digestion (18, 20, 21) and undermethylated (18, 23). Interestingly, although only one C_μ allele is functionally rearranged and transcribed from a V_h promoter, both C_μ alleles exhibit altered sensitivity and methylation. Chromatin structure of C_κ genes also changes in pre-B cells. In the pre-B cell line 70Z/3 one C_κ gene is germline and the other is rearranged, although neither is transcribed (25). Both C_κ alleles in 70Z/3 cells are sensitive to nuclease digestion (26).

The excluded C_μ and C_κ alleles are often transcribed from cryptic promoter cells as discussed below. However, it has been shown clearly that the transcription rate from the cryptic kappa promoter is several-fold lower than the rate from V_κ promoters (27); by analogy one may expect that cryptic C_μ transcription occurs at a lower rate than transcription from V_h promoters, although this has not been shown. The changes in nuclease sensitivity and methylation of C_μ and C_κ gene segments are apparently

independent from rearrangement with V regions and from high transcription rates; this has led to the speculation that the altered chromatin structure of these genes is mediated by their associated enhancer sequences and is then stabilized by demethylation (28). Although a role for enhancer elements in mediating changes in C_μ and C_κ chromatin structure remains to be established, clearly the changes are specific to the constant regions themselves.

The chromatin structure of C gene segments in B-cell lymphomas and plasmacytomas is similar to that in pre-B cells. In κ-producing cells both C_κ alleles are nuclease sensitive (21, 22) and display an absence of normal nucleosomal structure (29). As B cells begin to undergo isotype switching, other C_h genes become undermethylated on both chromosomes (24). IgA producer M603 contains three forms of C_α: a productively rearranged and expressed α; a deletion of the expressed α gene; and a C_α rearranged with *c-myc* at the switch region (30, 31). All three C_α alleles are sensitive to DNase I even though the enhancer region is missing from the C_α that is rearranged to *c-myc* (E. Kakkis, M. Mercola, J. Prehn, K. Calame, submitted). This suggests that the enhancer may not be involved in maintaining DNase I sensitivity. Thus, active chromatin structure, as evidenced by nuclease sensitivity and hypomethylation of C gene segments in pre-B cells, B-cells, and plasmacytomas, appears to be lymphoid and C-region specific but independent of rearrangement to V gene segments, transcriptional state, presence of enhancer elements, or of the final fate of the allele in terms of its functionality.

V-gene segments display a pattern of chromatin structure changes distinct from that of C-gene segments. Germline V-gene segments in lymphoid cells remain insensitive to nuclease digestion until they are rearranged to J-gene segments (19–22). After joining, V-gene segments acquire the same pattern of DNase sensitivity and undermethylation observed for C-gene segments (18, 22, 19). This alteration correlates with transcription of the rearranged V-gene segment and the lack of transcription of germline V-gene segments (32). Aberrant rearrangements of *c-myc* to C_α result in altered chromatin structure of the *c-myc* gene in a manner analogous to the effect seen on V genes in normal rearrangements (E. Kakkis, M. Mercola, J. Prehn, and K. Calame, submitted). Thus it does appear that the characteristic active chromatin structure of C-gene segments in lymphoid cells is acquired by genes juxtaposed to them.

STERILE TRANSCRIPTS Transcription is correlated with the observed changes in chromatin structure for immunoglobulin C gene segments in lymphoid cells. Normal rearrangement results in transcription from V promoters, but germline or aberrantly rearranged C gene segments are also

transcribed from apparently cryptic promoters to yield transcripts lacking V regions; these have been called "sterile transcripts." Thus the presence of sterile transcripts may be considered to indicate transcriptional activation of the C-gene segments, independent of V-gene promoters. Sterile C_μ transcripts have been found in a variety of lymphoid and related cell types including T cells (33–35), Abelson virus transformed pre-B cells (33, 34, 36), normal pre-B cells (36), myeloid lines (33), and B-cell hybridomas (37, 38). The cryptic promoters for sterile μ transcripts have been mapped 5′ to joined D regions (34, 38) and within (37), or 3′ to the J_h region (37, 38). Sterile C_κ transcripts of 8.4 kb are found in B cells that contain a germline C_κ (39, 40) and initiate from a cryptic promoter located about 8 kb 5′ of germline C_κ-gene segment (27).

There is only indirect evidence for sterile lambda transcripts. Hollis et al (41) have described a pseudo lambda gene which appears to have resulted from reverse transcription of an mRNA because it lacks intervening sequences and contains a poly A region 3′ to the coding sequences. However, rather than V_λ sequences, the pseudo gene contains flanking sequences found upstream of J_λ. Thus if the reverse transcription model for formation of this pseudo gene is correct, it implies that the gene arose from a sterile lambda transcript.

HYPERSENSITIVE SITES DNase I hypersensitive sites, which may indicate regions where particular nonhistone proteins bind, have been found in both active mu and kappa genes. The mu switch site (S_μ) is hypersensitive in B cells (20) and T cells (42). Originally the T-cell hypersensitivity was interpreted to indicate that the S_μ site might also be rearranged in T cells, leading to expression of T-cell receptors. However, the finding that T-cell receptor genes are not physically linked to immunoglobulin genes (43) makes this interpretation unlikely, and it is difficult to understand the significance of the S_μ hypersensitivity in T cells. In B cells, the heavy chain enhancer region is also hypersensitive (20). Several hypersensitive sites have been mapped for active kappa genes in kappa-producing myelomas: one near the V_κ promoter and two in the J_κ–C_κ intervening sequence, one at the enhancer and one 5′ of the enhancer (19, 29).

An especially informative situation occurs in 70Z/3 cells where one C_κ allele is functionally rearranged but not expressed (25). Upon treatment of the cells with lipopolysaccharide (LPS), the rearranged kappa gene is transcribed and a DNase I hypersensitive site appears at the C_κ enhancer region (44, 45). In fact, the germline C_κ as well as the rearranged C_κ enhancer acquires hypersensitivity upon LPS treatment and the 8.4 kb sterile kappa transcript is also induced (46), suggesting that the effect is enhancer rather than V_κ specific.

Changes in the Chromatin Structure of Immunoglobulin Genes May Precede DNA Rearrangements

VARIABLE REGION JOINING Changes of chromatin structure evidenced by increased sensitivity to nuclease digestion, demethylation, appearance of sterile transcripts, and appearance of hypersensitive sites have been shown to occur for C-gene segments in the appropriate B-cell environment, independent of rearrangement. Several groups have suggested that this chromatin structure may be required to precede variable region joining (21, 27). Perhaps the "openness" suggested by sensitivity to nucleases allows enzymes involved in DNA rearrangement access to appropriate recognition sequences flanking V, D, and J gene segments. Studies in which transfected genes are joined in vivo after introduction into lymphoid cells are beginning to illuminate this issue although there is currently no strong proof for the hypothesis. Joining occurs when only a few kb of flanking V_κ and J_κ sequence are transfected, thus ruling out a requirement for long-range effects of flanking sequences (47). However, activation of a co-transfected thymidine kinase gene correlates with DJ joining, which in turn suggests that local alteration of chromatin structure is correlated with joining (48).

The hierarchy of light-chain joining in which kappa genes rearrange before lambda genes (40, 49–51) and the fact that C_λ is not DNase sensitive in kappa producing cells (21) is also consistent with the suggestion that alterations in chromatin structure of C-gene segments precede joining. If chromatin changes do precede joining, alteration of C_μ chromatin would be the earliest known step in commitment to the B-cell lineage. However, since sterile mu transcripts have been found in T cells and myeloid cells, this early event may not irreversibly commit the cell to the B lineage.

ISOTYPE SWITCHING In plasmacytoma cells, isotype switching involves a recombination between S_μ and the switch site 5′ to the expressed C_h gene segment; C_μ and other intervening C_h gene segments are removed (2, 52, 53). However, expression of delta (54), epsilon (55), gamma 2b (56), and gamma 1 (57) have been reported to occur without detectable rearrangement of the switch site corresponding to the expressed C_h-gene segment and without deletion of C_μ. Prompted by the lack of evidence for C_h rearrangements, it has been suggested (53) that long heavy-chain transcripts that begin at the V_h promoter and proceed down the unrearranged C_h region through the expressed C_h gene segment are spliced to produce the appropriate heavy-chain mRNAs. It has further been suggested that transcriptional activation and transcription through the heavy-chain region or through a portion of it may be an early, mandatory event prior to DNA rearrangement of C_h-gene segments (53).

There is firm evidence from nuclear runoff experiments for a long transcript containing C_μ and C_δ (58, 59). However, the putative longer transcripts, which for epsilon or alpha mRNAs would be more than 180 kb long, have never been observed. Clearly, technical limitations may prevent direct validation of such long transcripts, or they may be spliced during transcription. However, strong genetic and DNA-structural evidence exists for transcription units in the size range of 80–110 kb in the ultrabithorax (60) and antennapedia (61, 62) loci of *Drosophila.*

In support of the hypothesis that transcriptional activation must precede switch recombination, it has been shown that demethylation of C_h gene segments accompanies switching (23, 24), although no studies on the DNase sensitivity of downstream C_h genes in various B-cell types have been reported. Reports of transcripts isolated from normal B cells indicate they contain both alpha and gamma sequences, although it was not shown whether they contained V_h sequences; their size was only about 10 kb (G. Woloshak, T. Tomasi, C. Liarakos, submitted). C_α transcripts originating from cryptic promoters on the excluded allele (which is translocated to *c-myc*) have also been noted (63, 64), indicating transcriptional activation of this allele similar to that in M603 (214). The strongest evidence to date in support of the hypothesis, however, is from recent work by Stavnezer and coworkers with a subclone of the I.29 line (65). This line expresses only IgM and does not switch during passage in animals or in vitro. However, upon stimulation with LPS the cells begin to express IgA or, in a minority of cases, IgE. This thus represents a line committed to IgA expression, although it does not switch to IgA expression until it is stimulated with LPS. Stavnezer et al report that the C_α and C_ε gene segments are transcribed prior to LPS treatment and switching (65). This cell line will provide an ideal system for more detailed analysis of the chromatin structure and transcriptional state of C_h genes before isotype switching, so that definitive evidence for or against the transcriptional-activation model may be available soon.

TRANSCRIPTIONAL REGULATION OF IMMUNOGLOBULIN GENES

Steadystate levels of mRNA can potentially be regulated by differential transcription initiation, RNA splicing, polyadenylation, rates of processing and transport, or stability in the cytoplasm (66, 67). However, for the eukaryotic genes studied to date, the majority are primarily or exclusively regulated by modulation of transcription initiation, which determines transcription rate (66). This general pattern holds for immunoglobulin genes where lack of transcription initiation accounts for restriction of their

expression to the B-cell lineage (32) and differential transcription rates occur in B cells at different developmental stages [(58, 59); G. Lamson, M. Koshland, submitted].

Transcription Initiation Regions

DNA SEQUENCE OF TRANSCRIPTION INITIATION REGIONS Several light and heavy chain transcription initiation sites have been carefully mapped using S1 nuclease and primer extension techniques; others have been inferred from sequence analyses. These include: human kappa (68), murine kappa (69–76), murine lambda (77, 78), and murine heavy (37, 79, 80). As illustrated by Wall & Kuehl (1), both light and heavy chain transcription initiation regions contain sequences typical of eukaryotic genes at the initiation or cap site and at the TATA region, which is located about 25 bp 5′ to the initiation site and is required for correct positioning of polymerase (81). Thus, initial analyses of V-gene transcription initiation regions showed them to be similar to those of other eukaryotic genes.

Recently, as more V-gene transcription initiation regions have been sequenced, it has been noted that there are short sequences that are present in all of them. A unique and highly conserved octanucleotide sequence, ATTTGCAT, was noted by Parslow et al 5′ of all light chain V genes that have been sequenced (69). Intriguingly, the inverse of this sequence, ATGCAAAT, occurs 5′ of all sequenced V_h genes [(69); and S. Crews and G. Siu, personal communication]. Strong conservation of this sequence suggests that it may play an important role in transcriptional regulation of immunoglobulin genes. This suggestion was confirmed by recent experiments of Zachau et al (82) who constructed mutants of an active kappa gene which were deleted in the 5′ transcription initiation region. They found, upon transfection into plasmacytoma cells, that the region between −90 and −160 bp upstream of the initiation site was required for transcription from the V_κ promoter. They identified two sequences within this region that are conserved among V genes: the octanucleotide previously identified by Parslow et al (69), and a pentadecanucleotide, TGCA$^{G}_{C}$CTGTGNCCAG. The latter is less well conserved but is found upstream of other V_κ and V_λ genes. More recently, Bergman et al (215) have tested the effect of 5′ deletions on a V_x promoter and have located the required sequences between positions −69 and −104. This region contains the octanucleotide but not the pentadecanucleotide, thus suggesting that the conserved octanucleotide may be an important regulatory element for immunoglobulin transcription.

It is now important to define precisely the required sequences and to learn if they interact with enhancer elements or if they play a role in modulating transcription during B-cell development. The conserved

sequences may provide a tag for V genes that might be recognized by regulatory factors during B-cell development. If the same protein recognizes the octanucleotide in either orientation, this could also mediate coordinate regulation of heavy- and light-chain transcription rates. There are data showing that heavy- and light-chain transcription is indeed coordinately increased in response to growth and maturation factors (83, 84). Furthermore, in other systems growing evidence suggests: Common sequences 5′ of coordinately regulated genes such as the fibrinogen genes (85); and factors that bind only to the regulatory regions of particular genes such as *Drosophila* heat shock genes (86), glucocorticoid inducible genes (87), SV40 early genes (88), and the β-globin gene (89).

FUNCTIONAL STATE OF TRANSCRIPTION INITIATION REGIONS Strikingly, V-gene transcription initiation regions in their germline form are transcriptionally silent and become transcriptionally active only after rearrangement to J or DJ gene segments. Even in plasmacytoma cells, unrearranged V genes are not transcribed (32). Thus, the mechanism responsible for activating rearranged V genes works in *cis* rather than by a soluble, *trans*-acting factor. No sequence differences were observed in a 600-bp region 5′ to the transcription initiation site between a germline and an active V_h gene (37); therefore, the *cis*-acting alteration does not occur in the primary structure of the transcription initiation region. Furthermore, transcription initiation regions from germline V genes act to initiate transcription in in vitro systems and when injected into *Xenopus* oocytes, thus confirming that the primary DNA sequences required for initiating transcription are present (68). These results point to distant elements that must affect the transcription initiation region and are required for transcription of V genes in vivo. Transcriptional activation upon joining may be due to the functional proximity of elements such as enhancer sequences, discussed below, or to acquisition of an open chromatin structure as previously discussed, or to both.

In addition to activation associated with variable region joining that appears to be due to *cis*-acting effects, V-gene transcription rates appear to be affected by developmentally regulated soluble factors that are modulated in response to mitogens, and to growth and maturation factors for B cells. This regulation is discussed below.

Transcriptional Enhancer Elements

Transcriptional enhancer elements were first identified and studied in viral systems. The 72-bp repeat in the origin region of SV40 virus was shown to be required for transcription of SV40 early transcripts (90). Subsequent studies indicated that this region had the unusual property that its enhancing

effect on transcription was independent of its orientation with respect to the promoter or its distance (over a few kb) from the promoter. The element also worked only in *cis*, i.e. when it was on the same DNA molecule as the affected promoter (91). Further, the 72-bp sequence could activate transcription of heterologous, nonviral genes such as the rabbit β-globin gene (92). Elements with similar properties have been found in many other DNA and RNA viruses, especially in the long terminal repeat regions of retroviruses (93). Recently, growing evidence suggests that *cis*-acting elements with properties similar to the viral enhancer elements may also regulate cellular genes. In addition to the immunoglobulin enhancers discussed below there have been reports of enhancer-like elements in the MHC class II Eβ gene (S. Gillies, V. Folsom, S. Tonegwa, submitted), the insulin and chymotrypsin genes (94), sea urchin histone genes (95), and ribosomal genes (96).

IDENTIFICATION OF IMMUNOGLOBULIN ENHANCER ELEMENTS Several studies have thus predicted the occurrence in immunoglobulin genes of a *cis*-acting element capable of working over a distance at least as long as that from the transcription initiation site to the 3′ boundary of a V gene (about 500–700 bp) (32, 37, 89). Several groups using two fundamentally different and complementary approaches, simultaneously described a transcriptional enhancer element in the immunoglobulin heavy-chain locus located in the intervening sequence between J_h and C_μ. Banerji & Schaffner (97) and Mercola et al (98) made constructs in which the SV40 early or rabbit β-globin promoters, which require enhancers for efficient transcription, were cloned with portions of active heavy-chain genes. The vectors were transfected into a variety of cells and transcription from the SV40 or globin promoter was measured. Both groups identified a region located between J_h and C_μ that was capable of replacing the SV40 72-bp repeat as an enhancer for SV40 and β-globin promoters. Banerji & Schaffner (97) delimited the region of enhancer activity to a 1.0-kb Xba fragment; smaller fragments had partial activity. Subsequently, complete scanning of the region extending from 3 kb 5′ of a joined V_h gene into the second C_μ exon showed this to be the only detectable enhancer region (M. Mercola, J. Goverman, C. Mirell, K. Calame, now 216). The effect of the J_h–C_μ enhancer was independent of distance or orientation but required in *cis*, so that it strongly resembled the viral enhancer elements (97, 98, 99, 99a).

Gillies & Tonegawa (99) and Neuberger (99a) used an alternate approach to search for enhancer elements in the heavy chain locus. First Gillies & Tonegawa (99) transfected a complete γ2b gene into plasmacytoma cells and showed that in stably transfected lines the γ2b gene was expressed from its natural transcription initiation site. Subsequently they constructed

several mutant γ2b genes containing deletions in the J_h–C_μ intervening sequence and isolated stably transfected cells containing these genes. Mutants lacking the Xba 1.0-kb fragment were unable to transcribe the gene. The sequences contained in this fragment were thus required for transcription from a heavy chain promoter. In addition, the Xba 1.0-kb fragment had characteristics of a viral enhancer sequence because they could place it 5′ of the V gene, in either orientation, and restore proper levels of transcription.

Taken together, the experiments using both heterologous and V_h promoters provide strong evidence that the Xba 1.0-kb fragment located 5′ of S_μ in the J_h–C_μ intervening sequence has enhancer activity and activates joined V_h promoters. More recently an analogous region of the human J_h–C_μ region demonstrated enhancer activity assayed by transient transfections using the SV40 early promoter (100) or a V_h promoter (101).

Similar approaches have been used to search for an enhancer element within the J_κ–C_κ intervening sequence. Queen & Baltimore (102) reported that mutant kappa genes, lacking the region 3′ to the Hind III site in the J_κ–C_κ intervening sequence, did not transcribe normally when transfected stably into plasmacytoma cells. Subsequently this group made smaller deletions and showed that a region within the intervening sequence, which coincides with the previously identified DNase I hypersensitive site (19, 44), was required for transcription of kappa genes transfected into plasmacytoma cells (103). In their system the C_χ enhancer was about one third to one half as potent a transcriptional activator as the heavy chain enhancer. Picard & Schaffner (45) also demonstrated the presence of an enhancer element within the J_κ–C_κ intervening sequence by testing the ability of kappa sequences to enhance transcription from the rabbit β-globin promoter; a 475-bp region correlated with the DNase I hypersensitive site showed activity. However, these workers reported that the activity of the kappa enhancer was only 5% of the heavy-chain enhancer in their assay. Transfection experiments by Oi & Morrison (104) suggest there may be two regulatory regions within the J_κ–C_κ intervening sequence, which might correspond to the two DNase hypersensitive sites previously mapped there (19). Thus, although there is at least one enhancer element in the J_κ–C_κ region, there remain uncertainties about its strength and whether there may be a second regulatory region.

Regions of J_λ–C_λ intervening sequence have been assayed using the rabbit β-globin promoter but no enhancer activity was found (95). However, since this assay showed low activity with the kappa enhancer, a lambda enhancer may exist that was not detected. In addition to the obvious analogy to kappa and heavy-chain loci with respect to V-gene activation and rearrangement, other data indirectly suggest an enhancer

element in the lambda locus. Transfection studies carried out with a lambda gene introduced into nonlymphoid cells showed that correct transcription of lambda required the presence of the SV40 enhancer within 1 kb of the transcription initiation region (105). As discussed below, a putative lambda enhancer might not be active in nonlymphoid cells, but these experiments suggest that V_λ promoters do require an enhancer element. In other experiments, observed differences in the rate of synthesis of λ subtypes in inbred strains of mice could be explained by different strength enhancers associated with different C_λ gene segments (106) and a *cis*-acting regulatory gene responsible for inducibility of $\lambda-1$ bearing B cells has been mapped near the $C_{\lambda-1}$ structural gene, again suggesting a regulatory element (107). With techniques currently available for detecting enhancer elements, this question is likely to be answered in the near future.

LOCATION OF IMMUNOGLOBULIN ENHANCER ELEMENTS It is important to note that the location of both the heavy-chain and kappa enhancers is consistent with their proposed role in activation of rearranged V genes. As shown in Figure 1 the enhancers are located 3′ to the J cluster so that they are within 2–3 kb of joined V-gene promoters. Since the heavy chain enhancer is 5′ of the mu switch site, S_μ, it is retained after switch recombination (3) thus removing the necessity for each C_h gene to have an enhancer element. As predicted by this reasoning, no additional enhancer element was found in the C_α-gene segment 3′ to S_α (E. Kakkis, M. Mercola, J. Prehn, K. Calame, submitted). Further support for the idea that variable region joining activates V-gene transcription by bringing enhancer elements into functional proximity with rearranged V genes is the finding that an unrearranged V_h gene does not have a detectable enhancer element located 3′ (M. Mercola, J. Goverman, C. Mirell & K. Calame, now 216). A remaining question, as shown in Figure 1, is the maximum distance over which immunoglobulin enhancers may work. For instance, might the V region located nearest to the joined V also be activated? Studies on V_κ genes suggest indirectly that the kappa enhancer may be able to work over 8 kb but unable to work over 21 kb. A cryptic promoter about 8 kb 5′ of the kappa enhancer is activated in unrearranged C_κ genes (27, 39) but a V_κ gene 8 kb 5′ to an expressed V was shown to lack DNase sensitivity and hypomethylation characteristic of the joined gene (18). However, it is not proven that these characteristics directly reflect enhancer activation.

SEQUENCE OF IMMUNOGLOBULIN ENHANCER ELEMENTS The DNA sequence of the immunoglobulin enhancer elements is not very instructive with respect to identification of sequences that might be typical of enhancers or might give a clue to their mechanism of action. This is not surprising, because the viral enhancer elements also show little sequence homology to

one another. Weiher et al have carried out extensive site-directed mutagenesis studies on the SV40 72-bp repeat and identified a small core sequence of eight nucleotides that appear to be critical for enhancer activity, although a single core sequence is probably not sufficient for enhancer activity (108). The mouse and human heavy-chain enhancer regions have at least three copies of this core sequence. The functional significance of the core

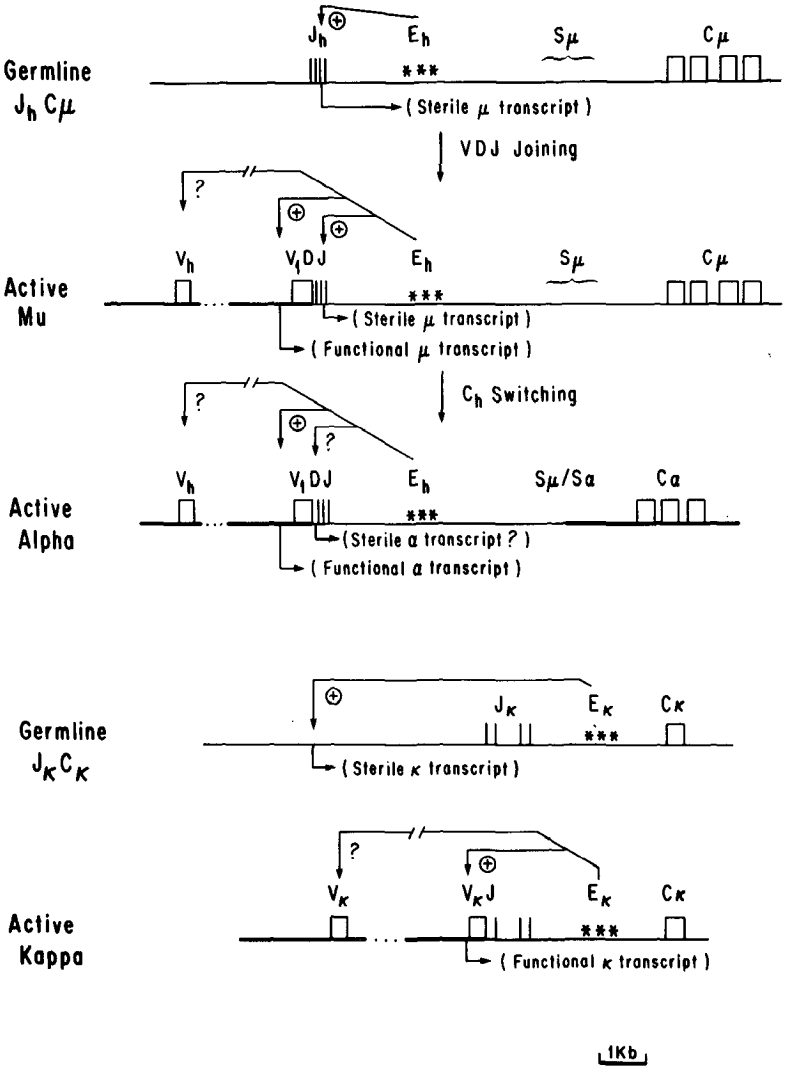

Figure 1 Location of immunoglobulin enhancers relative to V- and C-gene segments.

sequence is unknown and probably questionable since the kappa enhancer does not contain an SV40 viral core sequence, although it does contain short regions of homology to other portions of the SV40 72-bp repeat (45).

A much more significant aspect of sequence analysis is the finding that both heavy-chain and kappa enhancers share strong homology between the mouse and human elements (20, 101, 109). The homology in the heavy-chain enhancer includes but extends beyond the viral core sequences. This strong evolutionary conservation suggests sequences of functional importance that may be unique to each enhancer. Interestingly, there is no significant homology between the heavy-chain and kappa enhancers.

TISSUE SPECIFICITY OF IMMUNOGLOBULIN ENHANCER ELEMENTS One of the most significant aspects of the immunoglobulin enhancer elements which may illuminate not only important aspects of enhancer mechanism but also important new general mechanisms of eukaryotic gene regulation is the observation that the immunoglobulin enhancers works only in B cells (45, 97, 99). To provide an appropriate context for assessing the tissue specificity of immunoglobulin enhancers, it is helpful to consider first the restriction of immunoglobulin gene transcription to lymphoid cells. Many studies suggest that the restriction is not due simply to the fact that variable region joining occurs only in lymphoid cells—i.e. that rearrangement to functional proximity of an enhancer may be insufficient to activate transcription of V-gene promoters.

When somatic cell hybrids are formed between immunoglobulin secreting cells and nonlymphoid cells, the usual result is rapid and complete loss of immunoglobulin gene expression even though the chromosomes containing rearranged heavy- and light-chain genes are still present in the hybrid cells (110). This suggests that some soluble regulatory molecules are necessary for expression of joined V genes which may be diluted out or not expressed sufficiently in the hybrids. Similarly, the fact that in pre-B cell line 70Z/3 one kappa allele is functionally joined but not transcribed indicates that other factor(s) are required for kappa transcription that are missing in 70Z/3 cells (25). A number of recent cell transfection studies also suggest that a B-cell environment is required for high-level transcription of V genes because when functional, rearranged immunoglobulin genes are introduced by transfection into nonlymphoid cells, correct initiation of transcription is usually not observed (111–113; C. Mirrell, K. Calame, now reference 216) unless a viral enhancer is present (105, 114).

Clearly the most convincing evidence that unique factors are required for immunoglobulin gene transcription in B cells is the stringently specific expression of a rearranged immunoglobulin gene introduced into the germline of transgenic mice (115, 116, 217). Both kappa light chain and the

mu heavy chain genes are expressed in spleen at high levels, but not in testis, liver, kidney, heart, muscle, brain, or thyroid tissue (115, 116a). These elegant studies rule out any position effect for tissue-specific regulation of immunoglobulin genes and demonstrate that the few kilobases comprising the kappa and mu genes and their flanking sequence that were introduced are sufficient to respond to a lymphoid-specific environment and to confer tissue-specific regulation upon expression of the genes. Interestingly, however, when Grosschedl et al (116a) subfractionated lymphoid cells, they found that the foreign mu gene was expressed equally well in T and B cells. Thus both T and B cells appear to contain the factor(s) required for expression of joined immunoglobulin genes. The normal restriction of immunoglobulin transcription to B cells may result from lack of VDJ-joining in T cells.

Tissue specificity of enhancer elements could explain, at least in part, the tissue specificity associated with immunoglobulin gene expression. The initial reports of higher enhancer activity in lymphoid cells than in HeLa or in mouse L cells (97, 99) have held as more nonlymphoid cell types have been tested (M. Mercola, J. Goverman, C. Mirell, K. Calame, now ref. 216). Tissue specificity of enhancer activity has also been observed for viral enhancers where differences in enhancer activity are thought to be responsible for viral host range (117–119). Some viruses like SV40 are capable of infecting many cell types. Recently viruses which display strong tissue tropism have been shown to have enhancer elements which are much more tissue specific than the SV40 enhancer (120, 121). These observations are consistent with the idea that soluble factors, probably proteins, which recognize enhancer elements are required for their activity.

Scholer & Gruss carried out cotransfection experiments which confirmed that there are soluble factors that bind to the SV40 enhancer and are required for its activity (122). Recently a similar experimental approach showed that soluble factors which bind to the immunoglobulin heavy chain enhancer are also required for its activity. These experiments also demonstrated that the factor(s) required by the heavy chain enhancer are present in plasmacytoma cells but absent from nonlymphoid cells (M. Mercola, J. Goverman, C. Mirell, K. Calame, now ref. 216). Thus, the requirement of the heavy chain enhancer for factor(s) expressed only in lymphoid cells explains, at least in part, the tissue specificity of the enhancer and of immunoglobulin gene expression. Furthermore, it is easy to expand this type of regulation to a general scheme and to suggest that different cell lineages may express different subsets of enhancer factors, each factor capable of activating transcription of a single gene or set of related genes which contain the target enhancer sequence. Regulation of enhancer factor expression thus becomes another important control point in eukaryotic

gene regulation. With regard to immunoglobulin gene expression, it is interesting to speculate that pre-B and early B cells may express lower amounts of immunoglobulin enhancer factors than plasma cells where immunoglobulin gene transcription occurs at much higher levels. This hypothesis is currently being tested.

MECHANISM OF ACTION OF IMMUNOGLOBULIN ENHANCER ELEMENT Although a key to the role enhancer elements play in gene regulation is understanding their mechanism of action, this question remains unanswered for both immunoglobulin enhancers and for viral elements. Several models have been considered but little evidence supports them (93). It has been suggested that enhancer elements alter the chromatin structure of a large region which would allow the open conformation required for initiation of transcription. Enhancer elements may form Z DNA, but the heavy chain enhancer sequence does not have high Z-forming potential (97). Another suggestion for enhancer elements is that they act as an entry site for RNA polymerase II and/or other transcription factors. And finally enhancer elements may provide a site for anchoring genes to an appropriate place on the nuclear matrix for transcription.

Several new techniques should help in deciding this important question. Adenovirus Ela protein can replace the enhancer requirement of certain genes transcribed by Pol II (123) and by Pol III (A. Berk, L. Feldman, personal communication), which may provide an approach for analysis of enhancer function. In addition, if enhancer binding factors can be biochemically isolated this may help clarify the mechanism of enhancer action. Procedures have been used to purify other DNA binding proteins [(88); H. Weintraub, personal communication] that may also be successful for purification of enhancer factors. Finally, in vitro transcription systems are becoming more sensitive and may provide a means for dissecting the effects of enhancer elements.

LACK OF REQUIREMENT FOR HEAVY-CHAIN ENHANCER ELEMENT IN SOME B-CELL LINES Several variant B-cell lines have been described that have heavy-chain genes with deleted enhancer element but that appear to synthesize and secrete normal levels of heavy chains [(124); K. Rajewsky, S. Klein, F. Sablitzky, A. Radbusch, personal communication; L. Eckhart, B. Birshtein, personal communication]. This is contrary to the requirement found for the enhancer element in transfected heavy-chain γ2b genes (99, 99a). Wabl & Burrows found that an enhancer-deleted mu gene in an Abelson-virus–transformed pre-B cell line was also expressed at the expected higher levels in a pre-B $\times$ myeloma hybrid (124). They suggested that the identified immunoglobulin enhancer elements were only "transfection enhancers" and were not really required for expression of normal

immunoglobulin genes. At the present time it is difficult to evaluate these data or the possibility that identified enhancers are artifacts of the experimental system used to detect them, i.e. transfection.

If enhancer elements do not play a role in normal immunoglobulin gene regulation, it is difficult to explain the tissue-specific activation of rearranged V genes. However, it is also difficult to explain the apparent lack of enhancer requirement observed in the variant lines. It may be that deletion of the heavy-chain enhancer in the variants actually created another enhancer-like element. A similar phenomenon has recently been reported to occur with an enhancer-deleted SV40 virus (125). It is also possible that the chromatin structure of the heavy-chain gene once activated by an enhancer element "remembers" this alteration after the element is deleted, or that there are alternate and over-lapping mechanisms for activating V genes in B cells. This critical point deserves more study to establish the role of enhancer elements in normal immunoglobulin gene expression.

POSTTRANSCRIPTIONAL REGULATION OF IMMUNOGLOBULIN RNA

In this section other mechanisms that contribute to the overall regulation of immunoglobulin RNA levels in developing B cells are considered. Some are the same controls that operate on all eukaryotic mRNAs such as RNA processing, transport, and stability (66). However, since the heavy chain genes are complex transcriptional units, additional control points for these genes will be considered in more detail: the choice between membrane and secreted 3′ exons and the choice in expression of different C_h gene segments.

The Choice Between Mu and Delta

POLYADENYLATION The organization of the C_μ–C_δ region is:

$$5'C_\mu 1, 2, 3, 4\text{–}C_\mu S\text{–}C_\mu M\text{–}C_\delta 1, 2\text{–}C_\delta S\text{–}C_\delta M\text{–}3'$$ (2, 126).

In cells that express IgM and IgD on the surface, many studies have shown that the same V_h gene is used and that there is no rearrangement of C_δ, which is located about 2.5 kb 3′ to the C_μ membrane exons (54, 126–130). Runoff transcription in isolated nuclei, which measures polymerase loading, has confirmed the prediction that in cell lines that express both IgM and IgD (58) and in normal IgM–IgD$^+$ splenocytes (59) transcription occurs throughout the C_μ–C_δ region. Since the level of polymerase loading on C_δ is only 20–50% that for C_μ, it is proposed (58) that some transcripts may terminate between C_μ and C_δ and become mu mRNAs while the remaining V_h–C_μ–C_δ transcripts may be polyadenylated 3′ to C_μ or 3′ to C_δ. Appropriate splicing, determined by the site of polyadenylation, then

presumably generates mu and delta mRNAs. This model is consistent with the general finding that polyadenylation precedes splicing (66). Failure to observe the predicted 25-kb precursor containing both μ and delta sequences by Northern analyses of nuclear RNA led to the suggestion that splicing of δ transcripts may occur during transcription (58); however, technical artifacts like the lability of long transcripts or difficulty in transferring them to nitrocellulose have not been ruled out. Thus in IgM–IgD producing cells, delta mRNA appears to be produced as a result of differential polyadenylation and splicing.

In early cells that express IgM but no IgD, there is nevertheless transcription through the C_δ region (58). Therefore, in these cells, polyadenylation preferentially occurs after C_μ. Lack of delta precursors in nuclear RNA suggests that δ transcripts fail to be polyadenylated or else are rapidly degraded (58).

Blattner and Tucker have proposed a slight variation on the theme of differential polyadenylation (59, 126). They suggest a "gauntlet" model in which B cells at different developmental stages have varying levels of "endase" which clips and polyadenylates preferentially 3′ to the μS exon.

TRANSCRIPTION TERMINATION In IgM secreting cells no transcription through δ was found (58), suggesting that in these cells the synthesis of mu mRNA is determined by preferential transcriptional termination 5′ of C_δ. In Wehi 279, a B-cell lymphoma that represents a developmental stage just prior to IgM secretion, the termination mechanism probably also occurs since Rogers & Wall showed that C_δ is heavily methylated in these cells compared to IgM–IgD$^+$ cells (23). In the rare cases where IgD only is produced, there appears to be an illegitimate recombination that deletes C_μ (59, 131) even though there is no real switch region associated with C_δ (126, 131).

The Choice Between Membrane and Secreted Exons

The interesting observation that the heavy chain C_μ gene segment contains alternate exons, coding for a membrane and for a secreted carboxy terminus (132, 133), has been reviewed (1, 134, 135). Subsequent studies have shown that in fact all the C_h gene segments contain both membrane and secreted exons including: C_δ (136), human C_α and C_γ (137), C_α (138, 139), $C_\gamma 1$ (140), $C_\gamma 2b$ (140, 141), $C_\gamma 2a$ (142), $C_\gamma 3$ (143), and C_ε (144). The mechanism responsible for generating membrane and/or secreted mRNAs has been studied only for mu mRNA but it seems reasonable to assume that the same will hold for other C_h gene segments.

For mu, a mechanism of differential polyadenylation and splicing, rather

than transcription termination, is suggested by the appearance of both secreted (μS) and membrane (μM) precursors in Northern analyses of cells that are making predominantly secreted IgM (37, 58, 46). Further evidence against a termination mechanism was provided by the identification of a fragment corresponding to the spliced out membrane μ exons in IgM-secreting cells (145). Transfection of C_μ genes into a monkey kidney cell line, COS, demonstrated that these nonlymphoid cells have the ability to correctly polyadenylate and splice, producing both μS and μM mRNAs (146). In COS cells the choice of polyadenylation site was determined by proximity to the promoter because if the μM polyadenylation site was placed in the promoter proximal position it was preferentially used. However, it is unknown whether the same mechanism is used in lymphoid cells.

Cytoplasmic Stability and/or Processing Rates

Several findings suggest that the processing rate or cytoplasmic stability of immunoglobulin mRNAs may be regulated differentially during B-cell development. At the present, however, this evidence is indirect; studies that directly measure processing rate or mRNA half-lives in different cell types have not been reported. Mather et al found a 2–5-fold increase in C_μ transcription rate between pre-B lines and IgM secreting plasmacytomas (58). Since the difference in steady state levels is probably much greater although it was not measured in the same study, the data suggest differences in processing or stability. Similarly, G. Lamson & M. Koshland (submitted) find that upon LPS stimulation, there is an 800-fold increase in steady-state levels of mu mRNA in normal B cells that is not accounted for solely by transcription rate, suggesting differences in stability or processing.

Tucker et al measured transcription rates of delta RNA in normal B cells and found that the transcription rate of delta RNA: (*a*) is one half that of mu RNA, (*b*) doesn't change from neonatal to resting B cells, and (*c*) doesn't decrease after LPS stimulation (59). Since steady-state levels of delta mRNA are much lower than mu and decrease markedly during maturation and with LPS stimulation, regulation of processing or stability is implicated.

TRANSLATIONAL AND POSTTRANSLATIONAL REGULATION OF IMMUNOGLOBULIN SYNTHESIS

Translational Regulation

Although immunoglobulin mRNAs appear to be translated more efficiently than total cellular mRNA (1), there are few studies suggesting

translational regulation during development. Where translational regulation is suggested, posttranslational events could also explain the results, leaving it unclear whether regulation occurs during translation. An example is the synthesis of delta chains, which ceases completely after LPS stimulation, leading to the gradual loss of IgD from the cell surface (147). Runoff transcription studies show that LPS treatment does not alter the transcription rate of delta, suggesting posttranscriptional regulation, possibly at the level of translation (59). Studies by Lynch and coworkers described in the following section are also consistent with, but do not prove, translational control of alpha chain synthesis upon suppression by T cells.

Glycosylation

Glycosylation is known to be required in some systems for correct secretion or compartmentalization of proteins; for example, it is required for compartmentalization of mammary tumor virus glycoproteins (148). Cell surface expression of immunoglobulins does not appear to require glycosylation of heavy chains (1). The requirement for glycosylation in secretion of immunoglobulins varies with different isotypes: lack of glycosylation partially affects IgA secretion, does not affect IgG or IgD secretion, and is required for IgE secretion (1). For IgM, secretion in mature B cells is inhibited by tunicamycin, which completely inhibits glycosylation (149, 150, 151), although secretion is not inhibited by a new inhibitor that only inhibits terminal glycosylation of the core oligosaccharide (152). IgM secretion is not inhibited even by tunicamycin in earlier B cells (153, 154). Since many of these studies were reviewed by Wall & Kuehl (1), they are not described further here.

Recent studies increasingly suggest that glycosylation promotes proper folding of the polypeptide chains so that correct covalent assembly of light- and heavy-chains can occur (155, 156). For example, a variant MPC-11 line that makes an abnormally glycosylated heavy chain forms heavy-light dimers as well as the normal heavy-2-light-2 tetramers (156). Accumulation in myelomas of nonglycosylated intermediates which are abolished by treatment with tunicamycin suggests that glycosylation of heavy-chain polypeptides may be the rate-limiting posttranslational step in immunoglobulin synthesis (157). Since heavy chains particularly appear to be stabilized against degradation by assembly with light chains and since assembly appears to depend on proper chain folding induced by glycosylation, glycosylation could be a key regulatory step. However, there is currently no strong evidence for differential glycosylation during B-cell development, so whether this process is utilized for regulation remains a question.

Intracellular Degradation

COORDINATE REGULATION OF HEAVY- AND LIGHT-CHAIN LEVELS Older studies on myeloma proteins show that while many myelomas secrete only light chains or make excess light chains (158, 159), myelomas that make heavy chains in the absence of light chains are very rare. In the cases where they do, the heavy chains is not secreted (160–162). Gefter and coworkers studied an MPC-11 variant, producing a truncated alpha chain and no lambda chain, and found that the variant heavy chain was rapidly degraded. Upon fusion of this cell with an MPC-11 variant that made only light chain, the truncated heavy chain was assembled with light chain and secreted (163). Further work with this system has shown that rescue of the truncated heavy chain was due to association with light chain, not to a glycosylation defect in the original cell (164), and that the truncated heavy chain could also be rescued by transfection of the variant with a λ-2 gene (G. Sonenshein, personal communication). These data suggest that unassembled heavy chains are degraded rapidly, providing a simple means of equalizing heavy and light chain levels. There are also data to suggest that excess light chains may be degraded in some situations. In one MOPC 21 variant, excess light chains are degraded and fusion with a heavy chain–producing cell allows the excess light chains to be assembled rather than degraded (165).

More recently, studies by Lynch and his coworkers have strengthened the notion that light chains regulate levels of functional heavy chains. They have developed an elegant system for analyzing suppression using plasmacytoma M315 cells in culture. By immunizing mice under appropriate conditions with M315 protein, idiotype-specific suppressor T cells are made, which upon coculture with M315 cells cause suppression of IgA synthesis and secretion (166–170). They have shown that steady-state levels of λ-2 light-chain mRNA in M315 decreased, suggesting that a primary effect is on transcription or stability of λ-2 RNA. Steady-state levels of alpha mRNA are unchanged by coculture with the suppressor T cells. In spite of the remaining high steady-state level of alpha mRNA, the amount of alpha polypeptide in the cocultured cells is decreased to a level similar to the amount of lambda chains (166, 170). These results clearly point to a posttranscriptional regulation of heavy chains by light chains but do not yet point to a mechanism. Considering these and the MPC-11 studies together, there is a strong suggestion that heavy chains unassociated with light chains may be rapidly degraded intracellularly.

Unassociated heavy chains may be toxic to the cell (171), which would be consistent with lack of myelomas making heavy chain only. However, the

notion that unassembled heavy chains are degraded or are toxic to the cell is not supported by the fact that pre-B cells make cytoplasmic mu chains in the absence of any light chain expression (34). It is possible that the activity of degradative enzymes may change as B cells mature or that other controls remain to be elucidated.

PARTITION OF MU CHAINS DURING B-CELL DEVELOPMENT There is intriguing evidence that intracellular degradation accounts in part for the intracellular fate of μ chains in B cells at different developmental stages. It has been shown that both membrane and secreted forms of mu chains are present in pre–B cells and resting B cells (172) but their fates are different. In pre–B cells membrane mu ($_{\mu}$M) chains are degraded, but in resting and secreting B cells $_{\mu}$M chains are inserted into the membrane. Conversely, secreted mu ($_{\mu}$S) chains are degraded in pre–B and resting B cells, but are quantitatively secreted in secreting B cells (173). By using ionophores, which inhibit terminal glycosylation and transport but not covalent assembly of heavy and light chains, researchers have shown that stabilization of IgM against intracellular degradation is accomplished by assembly into monomers or pentamers rather than by glycosylation or cellular transport (173). In normal resting B-cells, which express both IgM and IgD on their surface, there is more translation of mu chains due to the higher steady-state level of μ mRNA. However, the turnover rate of IgM on the cell surface is also greater than that for IgD so that the final result is higher expression of IgD relative to IgM under steady-state conditions (147).

Allelic and Isotypic Exclusion

A unique feature of immunoglobulin genes among eukaryotic genes studied to date is the phenomenon of allelic exclusion—the observation that in any B cell only one of the two immunoglobulin alleles is functionally expressed. Allelic exclusion operates for both light and heavy chain loci. In addition, two light-chain classes, kappa and lambda, are encoded in unlinked families; isotype exclusion means that in a given B cell only a single light-chain family is functionally expressed.

Allelic exclusion and isotypic exclusion do not occur at the level of DNA rearrangement or transcription since for both heavy and light chains both chromosomes are often rearranged and may be transcribed (reviewed in 2). Analysis of normal and aberrant rearrangements in B cells at different developmental stages showed that in most cases only one rearranged chromosome was capable of making a functional polypeptide. A pattern emerged suggesting that the order of gene rearrangement was first, heavy, kappa next, and lambda last (49, 174–176). These studies and the stochastic model for isotype and allelic exclusion derived from them have been

reviewed recently by Coleclough (177). The stochastic model holds that rearrangements of heavy or light chain genes occur on one chromosome and then the other and continue until there is a signal to stop rearrangement. It further assumes that there is a high error frequency in the rearrangement events, so that the probability of a correctly joined gene is low, resulting in many nonexpressing cells, which presumably die. Recent work on the signals to halt rearrangement, which are key to accomplishing allelic and isotypic exclusion, are considered below.

HEAVY CHAINS Several groups have studied heavy-chain rearrangement in early pre-B cells represented by Abelson virus transformed lines. In these cells no light-chain rearrangement has occurred but both J_h alleles are usually rearranged with the nonexpressed allele often representing a DJ joining (34, 176, 178). There is no evidence of VD or DD joins (176). Thus, it appears that DJ joining occurs first and V-gene segments are joined later. A functional mu-heavy chain appears to signal a halt to V_hDJ joining (176, 178). Recently a variant of Abelson-transformed line 18–81 has been reported that makes μ- and γ-2b heavy chains from different chromosomes, suggesting allelic inclusion rather than exclusion (179). However, upon fusion with a light-chain producer, only the mu chain could assemble correctly with light chains. Thus, the requirement for signaling the recombinase system may be stringent and may require the ability of the heavy chain to assemble with light chains later in B-cell development. Further study of this or similar lines may help clarify the requirements for signaling heavy chain recombinases.

LIGHT CHAINS Many aberrant light chain RNAs and polypeptides are made in different cell lines, suggesting that combination of a functional light chain with heavy chain may be required to signal a halt in VJ joining (50, 175, 180, 181). Wabl & Steinberg suggest alternately that a heavy chain binding protein demonstrated in pre–B cells is replaced when a functional light chain is formed, and that this event signals termination of light chain rearrangement (171). Regardless of the exact nature of the signal, this general model accounts for both isotypic and allelic exclusion of light chains. Recently, Abelson virus–transformed bone marrow cells, which subsequently underwent VJ joining in culture, have provided two examples of clones expressing two functional kappa chains (S. Zeigler, O. Witte, personal communication). Further work is necessary to determine if this example of allelic inclusion is a characteristic of rearrangement in culture or if it is representative of normal VJ joining.

It may be difficult to advance our understanding of the exact nature of the signals that regulate V(D)J joining further until we begin to understand enzymology of the rearrangement events themselves. Unfortunately this

pivotal issue remains poorly understood at the present. However, recently artificial constructs of gene segments were correctly joined after transfection into pre-B cells (47, 182), and correct cutting at the boundary of a J segment occurred in vitro (H. Sakano, personal communication). Such systems should allow the nature of the recombinase enzymes and their regulation to be elucidated.

FACTORS THAT AFFECT IMMUNOGLOBULIN GENE EXPRESSION

A complex array of factors produced by other cells or by foreign organisms are known to regulate B-cell development and to modulate immunoglobulin gene expression. The biology and biochemistry of these factors are beyond the scope of this review and have been reviewed elsewhere (72, 183–185). This brief discussion focuses only on factors where the ultimate molecular level and/or mechanism of their effects on immunoglobulin gene regulation has been studied. There is enormous complexity in the regulatory network between B cells and T cells. It is exciting that many T-cell factors are now either being purified from clonal T-cell lines (186–188) or, their genes having been cloned, are being produced by recombinant bacteria (189). This provides an opportunity to study their mechanism of action on clonal B cells and to begin to unravel a very complex cellular regulatory network at the molecular level.

Bacterial Lipopolysaccharide—Multiple Effects

Bacterial cell-wall lipopolysaccharide (LPS) is a complex molecule which induces polyclonal activation of B cells in addition to other effects on the whole organism (190). Although the exact chemical structure of LPS is not known, a smaller defined molecule, 2,3 diacylglucosamine-1-phosphate, has been shown to mimic the action of LPS on B cells (191). Recently considerable progress has been made in sorting out the different effects of LPS on B cells.

ALTERATIONS OF TRANSCRIPTION RATE Two groups have carried out studies to assess the steady-state levels and transcription rate of mu genes in normal splenic B cells after LPS treatment [(59); G. Lamson, M. Koshland, submitted]. Both find that LPS causes a large increase in steady-state levels of mu mRNA—as much as 800-fold was observed by Lamson and Koshland. Part, although not all, of this increase is due to an increase in the transcription rate of the mu gene (59). It is interesting that LPS treatment does not cause as great an increase in mu mRNA levels in B-cell lymphoma lines, presumably because their mu gene transcription is already activated

by an unknown mechanism, which may be related to their ability to grow continuously in culture (G. Lamson, M. Koshland, submitted; P. Tucker, personal communication). In BCL1 there is no increase in mu transcription rate after LPS treatment (P. Tucker, personal communication). LPS treatment does not usually alter the level of mu mRNA in Abelson transformed—B cell lines either. However, in a variant 18–81 Abelson-transformed line where the mu gene contains a deletion in the J_h–C_μ intervening sequence that correlates with loss of mu expression, LPS treatment restores mu gene expression (56). These data suggest that LPS treatment might replace or mimic the enhancer in activating V_h transcription, but alternate explanations are also possible.

The effect of LPS treatment on kappa transcription is also intriguing. When a rearranged kappa gene was transfected into the 18–81 line containing the deleted mu gene responsive to LPS treatment, the transfected kappa gene was expressed and the expression was stimulated upon LPS treatment (192), suggesting coordinate regulation of heavy and light chain gene transcription by LPS stimulation. These data do not rule out, however, the possibility that light chain induction was secondary to heavy chain induction. Studies on the 70Z/3 line suggest a direct effect of LPS on kappa transcription because, although one kappa gene is functionally rearranged in these cells, it is not transcribed until LPS treatment (25), and there is no effect of LPS on mu mRNA levels in 70Z/3 (153). Further, the LPS activation of kappa transcription correlates with the appearance of a DNase I hypersensitive site (44) at the kappa enhancer region (105). Since both the germline and rearranged kappa alleles in 70Z/3 are activated, the effect seems to be enhancer rather than V_κ specific (46). It is interesting to speculate that LPS may induce the synthesis of an enhancer binding protein required for activation of kappa transcription.

ALTERATIONS IN POSTTRANSCRIPTIONAL STEPS A variety of studies clearly point to posttranscriptional effects of LPS treatment on immunoglobulin gene expression, although at this time the data are too fragmentary to define a cohesive pattern. In BCL1, LPS treatment causes a 6–12-fold increase in muS and 3–4-fold increase in muM polypeptide levels, although the transcription rate of μ mRNA is unchanged (193). As noted previously, δ mRNA transcription in normal B cells is unchanged upon LPS treatment (59), but delta polypeptide synthesis is completely shut down (147). Mu polypeptide synthesis and terminal glycosylation are increased in 70Z/3 cells after LPS treatment (153) even though the transcription rate of μ mRNA is unchanged.

INDUCTION OF ISOTYPE SWITCHING There is evidence that LPS treatment can modulate isotype switching. A clear mechanistic understanding is

unfortunately limited by our poor understanding of the molecular events involved in the DNA rearrangement process (194). Rajewsky and coworkers (195) treated normal splenic B cells with LPS and noted that expression changed from IgM to IgG3. When IgG3 secreting cells were purified they found that DNA rearrangements and deletion of C_μ gene segments had occurred. Vitetta and her coworkers also noted that LPS treated cells expressed IgM or IgG3; they found that additional T cell–derived factors were necessary, however, for expression of other isotypes such as IgG1 (196, 197), so the LPS signal is probably only one of several involved in regulation of isotype switching. Recently, the I.29 subclone described by Stavnezer and her coworkers (65), which appears to be committed to IgA expression (possibly because it has already received other regulatory signals), was induced to actual expression of IgA with LPS treatment. This strengthens the idea of a role for LPS in isotype switching and promises that more detailed analyses of the LPS effect on isotype switching should appear in the future using this cell line.

T Cell–Derived Factors

GROWTH OR MATURATION FACTORS Several T cell–derived factors affect the growth or maturation of both T cells and B cells. For example, IL-1 induces kappa transcription in 70Z/3 cells (198), IL-2 functions as a differentiation factor late in B-cell development (199) and IL-3 supports the growth of early pre–B cells in vitro (200). A mitogenic peptide has been identified in the supernatant of a stimulated inducer T-cell clone which stimulates both B and T cells to divide (186). The overlapping effects of these and probably other T-cell factors on both cell types adds increased complexity to the T cell–B cell regulatory network.

Paul and coworkers have begun to identify specific regulatory steps at which different factors regulate development of resting, normal B cells (2, 199, 201). Cross-linkage of surface IgM receptors with anti-mu causes resting B cells to enter G1, but proliferation depends on the presence of BSF-p1 (formerly called BCGF). At this point Northern analyses show that the levels and ratio of $_\mu$M and $_\mu$S mRNA do not change significantly. However, upon addition of two T-cell supernatants, B15-TRF and EL-TRF, there is a 9-fold increase in the ratio of $_\mu$S to $_\mu$M and a 5–10-fold increase in overall steady-state level of mu mRNA (199). It will be interesting to learn whether these effects on mu mRNAs occur by alteration of transcription rate and differential polyadenylation as might be predicted from our current understanding of mu mRNA synthesis. They also found that kappa mRNA steady-state levels increased coordinately with mu mRNA levels (199). Similar results in a slightly different system have been

reported by Vitetta and coworkers (197, 202). T-cell supernatants cause increased secretion of IgM in BCL1 cells which correlates with an increase in the steady-state level of mu mRNA.

Sidman et al (83, 189, 203, 204) have characterized and partially purified several maturation factors from a cloned helper T-cell line. These factors cause a coordinate 18-fold increase in both heavy and light chain polypeptide synthesis in Wehi 279 cells and an increase in the ratio of muS to muM. The factors, one of which has been shown to be gamma interferon (204), also induce resting splenic B cells to active immunoglobulin secretion. Changes in steady-state RNA levels or transcription rates have not yet been reported for these factors.

ISOTYPE-SPECIFIC FACTORS There are many reports of T-cell factors that either induce B cells to switch to a given isotype (197, 198, 205–207), help B cells that have already switched to a given isotype (183, 205, 208, 209), or suppress B cells of a given isotype (167, 210, 211). Since many aspects of isotype switching are still controversial (167), it is difficult to study the mechanism of action of these factors at a molecular level. We have suggested (212) that some isotype-specific factors might ultimately lead to alteration in the chromatin structure of C_h gene segments but currently no data support this suggestion. This is an intriguing area for further work.

IDIOTYPE-SPECIFIC FACTORS Lynch and his coworkers have developed an elegant system in which they are able to analyze the effect of an idiotype-specific factor made by suppressor T cells on the synthesis and secretion of IgA in plasmacytoma M315 (166, 170). Coculture of M315 with enriched populations of anti-Id-M315 T suppressor cells results in a dramatic decrease in the synthesis and secretion of IgA. They have shown that the primary effect appears to be a decrease in the steady-state level of lambda mRNA, although they have not yet determined whether this is due to decreased transcription, processing or stability of the lambda mRNA. They find that both λ mRNAs in M315—λ-2 which is functional and λ-1 which is nonfunctional—are coordinately decreased in their steady-state amount. Alpha heavy chain is unaffected by suppressor cells in spite of the fact that the recognized idiotype is determined by the alpha heavy chain. The interesting coordinate regulation of heavy chain polypeptide levels by the light chain levels in this system has been discussed earlier.

Abbas and coworkers have also studied the idiotype-specific suppression of cultured M315 cells and have shown that when a M315 × MPC-11 hybrid line was cocultured with anti-Id-M315 T suppressor cells, only the M315 idiotype was suppressed (213). They did not analyze heavy- and light-chain mRNA levels, however, so it is not clear if kappa transcripts were also

suppressed. It should be interesting to transfect lambda and kappa genes into M315 and analyze their response to suppression. Clearly, clonal B-cell lines that respond in culture to specific T-cell signals provide an excellent system for continued elucidation of how T-cell factors regulate immunoglobulin gene expression.

THE FUTURE

"'Curiouser and Curiouser,' cried Alice" (Lewis Carroll, *Alice in Wonderland*).

It is evident that substantial progress has been made recently in the study of immunoglobulin gene regulation. An early role for chromatin structure has been suggested. Studies with transgenic mice and gene transfections have shown that immunoglobulin genes and their immediate flanking sequences are sufficient to encode information for tissue-specific expression. Intriguing conserved sequences have been found 5′ of V-gene transcription initiation sites. The importance of *cis*-acting elements has been clearly defined and transcriptional enhancer elements identified. An added mechanism for gene regulation is suggested by the evidence for tissue-specific expression of enhancer factors. Posttranscriptional controls have been more precisely evaluated and we are beginning to be able to analyze the molecular mechanisms employed by various B-cell mitogens and growth or maturation factors.

In the immediate future, several technical advances can be expected to accelerate progress: (*a*) Improved ability to introduce genes into cells using retroviral vectors or into whole animals using microinjection techniques, (*b*) new approaches for isolation and purification of DNA binding proteins, (*c*) cloning and large-scale purification of regulatory factors for B cells; (*d*) isolation of clonal B-cell lines that can differentiate in culture in response to defined developmental signals; and (*e*) in vitro systems for transcription, RNA splicing and, possibly, for V(D)J joining and isotype switching. As immunoglobulin gene regulation becomes well defined, it should serve as a paradigm for understanding the members of the other similarly complex supergene families capable of measured responses to diverse signals.

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Annual Review of Immunology
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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 197–212

IMMUNOTOXINS

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HISTORICAL PERSPECTIVES

The concept of delivering a toxic moiety by a carrier that binds specifically to a foreign substance in the body but not to host tissue can be traced back to Paul Ehrlich (1). Ehrlich proposed attaching toxic heavy metals to dyes that would bind specifically to infectious agents. In the 1950s, David Pressman suggested that antibody could act as such a carrier (2, 3). He coupled tumor-reactive antibody to isotopes and used these conjugates experimentally to determine the specificity of binding. In the past 25 years, many investigators have contributed insights into the mechanisms by which plant and bacterial toxins kill cells (4–13). Thus, in the case of diphtheria toxin, we know now that the toxic portion enters the cell cytoplasm by translocation across a membrane (14, 15) and enzymatically inactivates EF2 (16–19), thereby inhibiting protein synthesis and causing cell death (20). Studies of the plant toxin, ricin, suggested that it follows a similar intracellular pathway and also inhibits protein synthesis, albeit by a slightly different mechanism (11). These insights together with the development of methods for generating purified antibody (21) have made it possible in the last five years to prepare immunotoxins that kill cells in vitro with a high degree of specificity (22–28).

BIOCHEMISTRY OF TOXINS

A structural similarity in plant and bacterial toxins inhibits protein synthesis: they are usually heterodimers made of a polypeptide chain (B chain) that binds the toxin to target cells and a second chain (A chain) that displays enzymatic activity (11). The two chains are linked by a disulfide bond. Diphtheria toxin is a slight exception in that a single proteolytic cleavage is required to generate an A and a B chain (29) that are also

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disulfide bonded. In addition, it is provocative that the subunits of all the plant toxins have approximately the same apparent molecular weight (11, 12), about 30,000, that the A chains attack the 60S ribosomal subunit (11, 12, 30) and the B chains bind to galactose (11, 12, 30). Moreover, the A and B chains of abrin and ricin, two toxins derived from phylogenetically distant plants, can be interchanged to produce hybrid molecules of relatively high toxicity (11, 30). These observations suggest significant conservation in function and structure. Whether the structural conservation is at the three-dimensional level only or reflects primary amino acid sequence homologies remains to be determined. There is also a variety of plant toxins composed of A chains only, e.g., gelonin (31) and pokeweed antiviral protein (PAP) (11, 32). These A chains function in the same way as the A chains of intact toxins.

The complete amino acid sequence of ricin has been determined by Funatsu et al (33, 34). Stretches of hydrophobic amino acids have been identified in the A (residues 144 to 168) and B chains (residues 161 to 180 and 104 to 153). Both the A and B chains of ricin clearly have the capacity to penetrate into lipid bilayers (35, 36), presumably because of these hydrophobic regions. Ricin A chain has a mannose rich oligosaccharide (attached to amino acid 10) and the B chain has two such carbohydrate moieties (attached to amino acids 93 and 133) (33, 34). In both cases, the side chains are linked through N-acetyl glucosamine to asparagine (37, 38).

BIOLOGY OF TOXINS

In the case of ricin, the B chain is a galactose-specific lectin (12, 13). Hence, ricin binds to virtually all eukaryotic cells. The binding step is followed by endocytosis (30). Electron micrographs of endocytosed ricin in which detection was achieved by staining with colloidal gold particles labeled with specific antibody, have revealed ricin to be concentrated in both endosomes and phagolysosomes (F. Jansen, personal communication). Translocation of the A chain of ricin across a membrane of an endocytic vesicle is required for cell killing (30). Presumably, the disulfide bond between A and B chain is cleaved before translocation. It is not clear whether this translocation occurs from an endocytic vesicle or from a phagolysosome. If it can only occur from a phagolysosome, then there may be a very short period of time during which translocation can take place, i.e., before the A chain is degraded. The A chain is capable of translocation by itself (35, 36), but there is considerable evidence that the B chain can markedly enhance the rate of translocation, i.e., increase the rate of cell killing (39, 40). After entry of the A chain into the cytosol, the A chain catalytically inactivates the 60S

ribosomal subunits by binding at or near the EF2 binding site and thereby inhibits protein synthesis (11, 12, 30).

IMMUNOTOXINS

The strategy of preparing immunotoxins is to couple an antibody (or in some cases, an antigen) to the holotoxin or the A chain and thereby to create a hybrid molecule with the specificity of the immunological ligand and the toxicity of the toxin. There are two major types of immunotoxins: those containing the intact toxin and those containing the A chain derived from it (22–28). Immunotoxins composed of intact toxins, which must be used in the presence of galactose to prevent nonspecific binding, invariably show higher levels of target cell toxicity in vitro than those containing only A chain (41, 42). However, their nonspecific toxicity in vivo is so great as to preclude their use. In contrast, immunotoxins containing purified A chain have a very low nonspecific toxicity in mice (43) and yet some of them can be highly toxic to specific target cells both in vivo and in vitro (44, 42, 43). In vitro, the specific toxicity sometimes approaches the toxicity of ricin itself (42, 43).

One general method of preparing immunotoxins is to use a thiol-containing heterobifunctional crosslinker, e.g., SPDP, which attacks primary amino groups on the antibody and by disulfide exchange can attach either the SH-containing A chain or the SPDP-derivatized holotoxin to the antibody (45, 46). If the disulfide exchange is carried out at neutral pH, a relatively stable disulfide bond is formed and the conjugate remains intact when incubated with fresh mouse serum in vitro (K. Krolick, 1982, unpublished observations).

The nature of the linkage between the A chain and the antibody is of critical importance in determining toxicity. If the bond cannot be broken readily in an endosome/phagolysosome (43, 47), e.g., a stable thioether bond, then toxicity is virtually abolished (43). In contrast, if the bond is highly unstable, then the conjugate may dissociate either before it reaches the target cell or, perhaps, prematurely within the target cell. In the latter case, the A chain may be degraded before translocation can occur. Recent studies by Jansen (personal communication) have followed the fate of A chain from an immunotoxin in a T-cell line. They examined the fate of the A chain by electron microscopy after treating cell sections with colloidal gold particles bound to monoclonal anti-A chain antibody. When immunotoxins were incubated with cells in a normal medium, the endocytosed A chain proceeded rapidly from endocytic vesicles to phagolysosomes. If cells were cultured with ammonium chloride (which raises the pH of endocytic

vesicles and slows phagolysosome formation), the length of time before A chains entered phagolysosomes was increased five fold (F. Jansen, personal communication). It is known that ammonium chloride can potentiate the toxicity of ricin-A chain immunotoxins from 5–200 fold, depending upon the antibody and experimental conditions (48, 49). Thus, the rate of degradation of A chain may be a major factor in determining the toxicity of particular A-chain immunotoxins.

The specificity of killing by A-chain immunotoxins has been firmly established. For example, immunotoxins directed against the immunoglobulin idiotype or isotype of a murine B cell tumor (22, 44), or against a Thy 1 antigen on T cells or T-cell leukemias (42, 50, 51) are exquisitely specific in vitro. Immunotoxins containing intact ricin are specifically cytotoxic in vitro if a high concentration of galactose is present to inhibit the nonspecific binding by the lectin sites on the B chain (26, 52–55).

The killing efficiency of immunotoxins is usually measured by their capacity to inhibit protein synthesis by target cells bearing the antigen in question. More precise quantification can be obtained when cytotoxicity is measured by clonigenic assays (56) or by the *rate* of inhibition of protein synthesis (39, 41, 49). The latter presumably measures the rate of entry of A chain into the cytoplasmic compartment. When any of these measurements are applied to A-chain immunotoxins, there is marked variability in their potency. Some A-chain immunotoxins can mimic (42, 50, 57) the toxicity of ricin itself; other immunotoxins are only modestly toxic even at very high concentrations (42). Even when A-chain immunotoxins are highly effective, the kinetics of inhibition of protein synthesis is slower than when ricin is used (41, 42). The major variables appear to be the density of cell surface antigen (58), the rate of endocytosis of the antigen-immunotoxin complex (which in turn depends upon the nature of the target cell antigen) (49), the binding affinity and isotype of the antibody (49, 59), the stability of the linkage between A chain and antibody (43, 59) and the number of A chains per antibody molecule. The nature of the antigen and the linkage between A chain and antibody are particularly important factors.

BONE MARROW TRANSPLANTATION

The ability of immunotoxins to kill specific subsets of cells efficiently in vitro has led to their application in the deletion of particular cell types in suspensions of bone marrow cells (42, 51–53, 55, 56, 60). The ultimate objective is to facilitate bone marrow transplantation in the human as an approach to treatment of cancer and diseases of the hematopoietic system. Autologous bone marrow transplantation is used as an adjunct to treatment for certain types of cancer which are highly susceptible to X-

irradiation and/or chemotherapy (61, 62). The approach is to obtain bone marrow from a patient in remission (preferably in the first remission) and to freeze it. If the patient subsequently relapses, the patient is then subjected to "supralethal" therapy with X-irradiation and/or chemotherapy in order to eradicate the tumor. The patient is then rescued from death by infusion of his own bone marrow.

It would, of course, be highly desirable to purge such bone marrow of cancer cells by a cancer cell-reactive immunotoxin. The only requirement of such an immunotoxin is that it should not damage the stem cells which are needed to reconstitute the patient's hematopoietic system.

The first experiments to evaluate this approach in a model system utilized bone marrow from mice bearing the BCL_1 tumor in conjunction with an affinity purified rabbit anti-mouse Ig A chain immunotoxin (60, 44). In this model system, a single viable BCL_1 cell causes detectable tumor in 50% of adoptive recipients by 12 weeks. All lethally irradiated recipient mice were reconstituted successfully. Of animals receiving such treated bone marrow, 75% remained disease free. The other 25% subsequently developed evidence of disease. No evidence was obtained for escape of a genetic variant; that is, the tumor cells that grew out remained highly susceptible to anti-Ig A chain immunotoxins. Of particular interest was the finding that one animal developed leukemia at a time beyond that in which a single neoplastic cell causes detectable leukemia in a virgin animal, and a second animal that appeared disease free had leukemic cells in its tissues as shown by transfer of spleen cells from this animal into virgin animals. We interpreted the findings in these two animals as evidence for an acquired–host resistance to the tumor, perhaps an anti-idiotypic response. This response induced a state of relative tumor dormancy that lasted for at least a month in one animal and for at least four months in the other animal. These studies, therefore, emphasize the role of the host immune response in determining the fate of an animal with a relatively small number of tumor cells. Similar results were subsequently obtained in rats by Thorpe and coworkers (63). Based on the studies in rodents, Phase I trials have now begun in humans (64) and it appears that A-chain immunotoxins are not toxic to transplant recipients. Long term followups will be required to determine whether such immunotoxin, treatment of bone marrow will improve therapy. Interpretation will be difficult if in vivo therapy is unsuccessful.

A variation on this theme is to eliminate T lymphocytes from allogeneic bone marrow. Such bone marrow, of course, does not contain tumor cells, but mature T cells in the bone marrow are responsible for the high incidence of fatal graft versus host disease (GVHD) in such transplant recipients (61). Vallera and his coworkers (52) have reported prevention of GVHD in allogeneic bone marrow transplants in mice, and Filipovich et al (53) have

performed HLA-matched bone marrow transplants in humans following treatment of marrow with *ricin* immunotoxins specific to T cells in the presence of galactose.

MODULATION OF THE IMMUNE RESPONSE

It would be attractive to use immunotoxins in vivo to remove a subset of lymphocytes transiently in order to increase or decrease a particular type of immune response. Thus, in the case of cancer and certain infectious diseases, increases in certain types of immune responses would be desirable whereas in autoimmunity or immunologic deficiency disorders caused by T cell–induced suppression, it would be attractive to decrease the responsiveness of particular lymphocyte subsets. Two lines of experimentation have taken place with these objectives in mind. In vitro, there are a number of reports of induction of immunologic tolerance at the B-cell level to protein antigens coupled either to intact ricin (65) or to its A chain (44). If this approach can be utilized in vivo, it might be useful for preventing antibody responses to the murine monoclonal antibodies when subsequently administered for therapeutic or diagnostic purposes.

The use of anti-δ A chain immunotoxin in vivo which deleted virgin B cells (44) is a different type of experimental approach for altering immune responsiveness. Normal or above normal levels of IgD^+ cells were present 14 weeks after treatment (66). These cells had presumably been generated from stem cells, pre-B cells and IgD^- IgM^+ B cells. Thus, subsets of lymphocytes can be transiently deleted by this approach. Immunotoxins directed against subsets of T cells would be attractive candidates for modulating the immune response. At the present time, however, there is insufficient information about the cellular components of the immune response and their precise role in generating effector mechanisms to design appropriate strategies for particular diseases.

IN VIVO USE OF IMMUNOTOXINS

Current research has two major objectives: To use these reagents to eradicate cancer, and, as mentioned above, to modulate the host immune response. The requirements for effective use of immunotoxins in vivo are considerably more formidable and stringent than the ex vivo use described above for bone marrow transplantation. Potential problems include nonspecific toxicity due primarily to damage to the reticuloendothelial system (RES) (67–71); specific toxicity due to cross reactivity of the antibody in the immunotoxin with antigens present on life-supporting normal tissue; interaction of immunotoxin with tumor associated antigens

released into the circulation thereby forming antigen-antibody complexes; penetration of the immunotoxin into neoplastic tissue or into body compartments where there is a blood tissue barrier; and immune responses to the immunotoxin.

The problem of cross reactivity between monoclonal antibodies raised against tumor-associated antigens and antigens present on normal cells may represent the major obstacle. Evidence increases that tumor-associated antigens are, for the most part, not tumor specific and probably represent differentiation antigens present on subsets of cells in the organ giving rise to the tumor and, not infrequently, cells of other tissues as well (72–74). It will be of critical importance to evaluate, therefore, the extent of these cross reactivities to determine if a particular immunotoxin can be administered.

There are relatively few reports of experimental use of immunotoxins in vivo. Moolten et al (75) described the effective use of tumor-reactive diphtheria toxin conjugates in an SV-40 induced tumor model. The use of immunotoxins containing the holotoxin is not a promising pathway because of the marked nonspecific toxicity in vivo of such conjugates. We have described the successful treatment of mice that were bearing enormous tumor burdens of the BCL_1 leukemia (20% of body weight) with anti-idiotype-A chain and anti-δ A chain immunotoxins (66). We determined that if nonspecific cytoreduction (both total lymphoid irradiation and splenectomy), reduced the tumor burden at least 95%, then administration of immunotoxins in three of four experiments was successful in inducing prolonged remissions in the tumor-bearing animals. In one such remission in which the animals were followed for 28 weeks and appeared to be disease free, tissues from all the treated animals transferred tumor into normal recipients. Thus, a host response, possibly an anti-idiotypic response, was keeping a small number of tumor cells "in check." The precise number of tumor cells was not estimated in these transfer experiments.

In contrast to the above study, other recent reports involve the administration of tumor cells and immunotoxins within a very short interval of each other and frequently into the same body cavity, e.g., peritoneal cavity. The results showed significant, but usually incomplete inhibition of tumor growth (42, 76, 77).

POTENTIATION OF TOXICITY OF A-CHAIN IMMUNOTOXINS

The variability of target cell toxicity of A-chain immunotoxins and the finding that they usually kill more slowly than ricin (39, 42, 43) in vitro makes it desirable to develop methods to increase their potency. One approach already mentioned is the use of lysosomotropic agents such as

ammonium chloride or chloroquine (49, 78). These agents raise the pH of endocytic vesicles and may perturb membranes in additional ways. One of the effects of these changes is to decrease proteolysis of ingested immunotoxin. This may account in large measure for their marked potentiation of target cell toxicity. Thus, Jansen (unpublished observation) has shown by a clonigenic assay that A-chain immunotoxins can kill approximately 4 logs of leukemia cells. When ammonium chloride was added to the incubation medium, the number of cells killed was increased by 2 logs. It is of interest that potentiation is greater when the immunotoxin per se is less effective. The maximal increase of the toxicity of ricin by treatment of cells with ammonium chloride is two-fold. Perhaps, one of the major roles of the B chain of ricin is to protect the A chain from degradation. Regardless of mechanism, the B chain aids the translocation of the A chain and several strategies have evolved to exploit this role of B chain to potentiate the effectiveness of A-chain immunotoxin.

One approach to increase the potency of immunotoxins for in vitro use is to use "blocked"-ricin immunotoxins. By using a short crosslinker, Thorpe et al (79) have shown that some ricin-immunotoxin molecules will pass through a sepharose-asialofetuin column, indicating loss of galactose binding. It is presumed that the blockade of galactose-binding sites on the B chain may be a steric effect, i.e. the lectin sites on the B chain are "hidden" by the attached antibody. The result is the generation of a subset of immunotoxin molecules that retain the high potency of ricin immunotoxins but do not kill cells nonspecifically in the absence of galactose. Such immunotoxins are highly effective *both* in vitro and in vivo. They still retain significant nonspecific toxicity when used in vivo, however, probably because the RES removes such molecules from the circulation (67–69, 80).

Another approach is to add free B chains after target cells have bound A-chain immunotoxins (39, 40). Free B chains can bind to the A-chain immunotoxin more readily than they bind to the galactose-containing glycoproteins on the cell surface (40). Thus, they potentiate the toxicity of A-chain immunotoxins specifically in vitro where the concentration of extraneous glycoproteins is relatively low. This approach is unlikely to be useful in vivo, however, because of the very high concentration of galactose-containing glycoproteins and cells in the circulation. Thus the lectin binding sites on free B chains would cause them to bind to virtually all cells and serum glycoproteins and it is unlikely that such free B chains would reach the target cells coated with A-chain immunotoxin.

We have recently described the use of B-chain immunotoxins to potentiate killing by A-chain immunotoxins (81). The rationale was that if both immunotoxins were bound to the same cell, a portion of each would be endocytosed within the same vesicle. Therein, the A and B chains might

be cleaved from their respective immunotoxins, thereby allowing the B chain to potentiate translocation of the A chain. In vitro experiments in which immunotoxins of the same specificity were used showed a marked specific potentiation of A-chain immunotoxin toxicity when B-chain immunotoxins were added simultaneously. The latter, by themselves, are essentially nontoxic. Furthermore, the attachment of B chain to antibody resulted in significant attenuation of the galactose binding site. Thus, synergy depended on specificity of the B-chain immunotoxin for the target cell. Moreover, *specific* synergy could be obtained only if both immunotoxins were affinity purified in order to remove trace amounts of free A and B chains. If *both* free chains are present, ricin will reform, resulting in nonspecific toxicity.

A variation of this approach is to generate a B-chain immunotoxin in which the antibody specificity is directed to the antibody of the A chain immunotoxin, i.e., a "piggyback" approach. This approach was equally effective (82). It also allowed the use of an F(ab′) A-chain immunotoxin to coat the target cells. This has a potential advantage for in vivo use. Thus, the F(ab′)–A chain immunotoxin should be removed relatively rapidly from the circulation (83) but retained on the target cell surface for prolonged periods of time because of its univalency. Thus, it may be possible to inject the B-chain immunotoxin after much of the F(ab′)–A chain has been cleared from the circulation. This maneuver might minimize nonspecific toxicity and yet allow the potentiation of specific target cell toxicity.

It is of particular interest that F(ab′)–A chain immunotoxins can be highly toxic. In an earlier report, such conjugates were 70-fold less potent than immunotoxins made with the intact antibody (59, 62). In contrast, while using Daudi cells we have recently observed approximately similar dose response curves for F(ab′) A-chain and IgG-A chain in which the antibody was specific for human Ig (82).

CLEARANCE OF IMMUNOTOXINS BY THE RETICULO-ENDOTHELIAL SYSTEM (RES)

One of the major obstacles in utilizing immunotoxins in vivo is their rapid clearance by cells of the RES. Thus, it has been shown that ricin itself causes gross damage to hepatic sinusoidal cells and to the white and red pulp of the spleen (67–70). The hepatic uptake is mediated by nonparenchymal cells which appear to have receptors that bind the carbohydrate portions of the toxin and thereby remove it from the circulation (80). Since ricin A and B chains are rich in mannose-containing oligosaccharides (37, 38) and cells of the RES express receptors for mannose, it is understandable that immunotoxins containing ricin A and/or B chains are rapidly removed from

the circulation. It would, therefore, be desirable to develop a strategy in which mannose-free A and B chains could be utilized in vivo. As the first attempt in creating such molecules, Thorpe et al (84), have removed the carbohydrate moieties from ricin by treatment with sodium metaperiodate and the reducing agent sodium cyanoborohydride at pH 3.5. Under these conditions, there is oxidative cleavage of mannose, and the reduced aldehyde groups thus formed are converted to stable primary alcohols. There is little or no formation of Schiff's bases. These modified ricin molecules were less rapidly cleared by cells of the RES but were 4–fold more toxic to the animals than native ricin. In contrast, the toxicity of such deglycosylated ricin on cultured cells was reduced by 10–fold. This reduction in toxicity in vitro is attributed to a change in the rate of translocation of A chain into the cytoplasm since both the galactose binding function of the B chain and the ability of the A chain to inhibit protein synthesis were unaltered. In a collaborative study (E. S. Vitetta & P. E. Thorpe, 1984, manuscript in preparation), the separated A and B chains from deglycosylated ricin have been utilized to determine whether they would kill target cells effectively in the "piggyback" approach. The results indicate that immunotoxins formed with the deglycosylated A and B chains are almost as effective as immunotoxins formed with native A and B chain in killing Daudi cells in vitro. Thus, the use of such deglycosylated ricin A and B chain-containing immunotoxins might avoid the problem of overly rapid clearance of immunotoxins in vivo by the RES.

MODIFICATION OF THE GALACTOSE BINDING SITE OF B CHAIN

As mentioned previously, the attachment of B chain to antibody greatly reduced its ability to bind to galactose. Hence, B-chain immunotoxins, were highly specific in vitro (81, 82). Nevertheless, B-chain immunotoxins still retain their capacity to bind to galactose-containing asialofetuin (E. S. Vitetta, 1984, unpublished observation). It is obvious that much of the B-chain immunotoxin would be removed in vivo by galactose-bearing cells and glycoproteins, thereby preventing the immunotoxin from reaching its target cells. For this reason, we have investigated ways of altering the galactose-binding ability of the B chain before attempting in vivo administration. Studies performed several years ago by Sandvig et al (85) suggested that iodination of intact ricin by the chloramine-T method resulted in loss of toxicity. It was unclear whether this loss of toxicity was related to iodination of the A and/or the B chains and to what extent each retained its normal function. We, therefore, conducted a series of experiments aimed at investigating the effect of chloramine-T mediated iodi-

nation or of chloramine T treatment alone of the B chain on its galactose binding ability (E. S. Vitetta & J. W. Uhr, 1984, manuscript in preparation). It was found that B chains treated with sodium iodide and chloramine-T or chloramine-T alone (under conditions in which the recovery of B chains was approximately 20–40%) had lost their ability to bind to galactose-containing asialofetuin. Furthermore, B chains could no longer form covalently-bound homodimers or heterodimers with A chains. These B chains also failed to potentiate the killing of Daudi cells by A-chain immunotoxins. Nevertheless, when such treated B chains were covalently coupled to the appropriate antibody and tested in the "piggyback" killing assay of Daudi cells, they could still potentiate killing albeit to a lesser extent than immunotoxins formed with native B chains. Moreover, the failure to couple modified B chains to antibody as effectively as native B chains could account for the decreased toxicity.

These studies have implications beyond their obvious practical use. First, they indicate that the galactose-binding capacity of the B chain is not required for the potentiation of toxicity induced by A-chain immunotoxin. Secondly, it appears that A and B chains do not have to form disulfide-linked dimers in order to kill cells effectively. These results suggest activity that different domains of the B chain are responsible for lectin and A-chain translocation, respectively. Hence, modification of B chains by genetic engineering may be the strategy of choice for the future.

CONCLUDING DISCUSSION

Cloning of the genes encoding ricin A and B chains is underway in several laboratories. Unpublished results (Weaver et al, personal communication) indicate that the genes are contiguous so that the resultant polypeptide chains are synthesized as polyprotein (86). High resolution x-ray crystallographic analysis of ricin is also taking place (87, 88; J. D. Robertus, 1984, personal communication). Assignments have been made for approximately 80% of the amino acid residues. The two lectin binding sites of ricin identified by their binding of galactose appear to be at opposite ends of the B chain. As soon as these sites have been definitively identified, it should be possible to perform site-directed mutagenesis, thereby to synthesize B chains without lectin activity but with translocating activity for A chain. Since the chains can then be synthesized without carbohydrate portions, the problem of the uptake of F(ab)–A chain conjugates by the RES because of mannose residues will be circumvented. Obviously, as we learn more about the mechanisms of A chain translocation, new possibilities arise for altering the A chain to make it more effective. In addition, it is possible that further improvements on nature can be made in the B chain. Indeed, other

molecules could conceivably be substituted for the B chain that might be more effective in facilitating A chain translocation. Thus, as new biological and structural information is accumulated and correlated, new macromolecules can be designed.

The generation and use of immunotoxins represents an interesting multidisciplinary problem. Clearly, immunology will continue to contribute in a major way to the investigation. A major gap in our understanding of immunotoxins resides in the domain of cell biology. In particular, the intracellular mechanisms involved in the endocytosis and degradation of immunotoxins and the translocation of the A chain into the cytoplasmic compartment are still poorly understood. More information is also required concerning the pharmacokinetics of immunotoxins but this information should be more readily obtainable than their intracellular life history. The problem of cross-reactivity of monoclonal antibodies directed against tumor associated antigens has already been emphasized as a potentially significant obstacle. Future developments will rely heavily on manipulating the genes encoding the A and B chains of ricin and other immunotoxins in order to generate chains that have the desired characteristics. Regardless, the progress made to date has established the effectiveness of using immunotoxins in bone marrow transplantation and has provided a foundation for in vivo clinical trials.

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CONTENTS

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IN VITRO ANALYSIS OF MURINE B-CELL DEVELOPMENT

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INTRODUCTION

The development of B lymphocytes provides a useful model system for the examination of factors that regulate growth and differentiation. The wealth of information on the molecular biology of immunoglobulin gene structure and expression (1, 2) coupled with the abundant supply of tumor models for various stages of B-cell development (3, 4, 5, 6) serves as a framework for studies on normal or cultured cell populations. The structure and expression of immunoglobulin genes at the DNA, RNA, and protein levels can be measured with great accuracy and discrimination. Many additional phenotypic markers, including other cell-surface antigens, can be similarly followed. Using such markers one can assess the developmental phenotype of a cell population or clonal cell lines before and after growth alterations. In order to examine such changes in detail, researchers needed to develop in vitro systems to grow B cells and their precursors.

The purpose of this review is to compare the range of in vitro assay and culture systems useful to evaluate different stages of B-cell development. A major future goal of this experimental approach is to reconstruct in vitro the entire pathway of B-cell development and functional responses. Special emphasis will be placed on long-term culture techniques that have the potential to provide biochemically useful numbers of cells.

Overview of B-Cell Maturation

It is convenient to consider the process of murine B-cell development in three phases (7, 8). The earliest stage of B-cell development involves

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commitment of pluripotent hematopoietic stem-cell elements to the B-cell pathway, rearrangement of Ig genes, and expression of cell-surface immunoglobulin to serve as the antigen-specific receptor. Next, cells capable of responding to antigen must migrate and accumulate in peripheral lymphoid tissues where they can be activated by antigen in concert with secondary factors. Finally, these selected and activated cells must both expand in number and secrete large amounts of immunoglobulin into the lymphatic and vascular spaces.

Commitment of stem cells probably occurs in the bone marrow of adult animals and in the liver and spleen of fetuses. The very early stages of this process are poorly understood owing to the heterogeneity of the cell populations and the lack of antigenic or molecular markers with which to isolate these very early cells. Recently, experimenters have characterized Abelson murine leukemia virus (A-MuLV)–transformed cell lines derived from placenta and demonstrated that these retain both mu-gene loci in the germline state (E. Siden, personal communication). Such lines may be analogs of very early B-cell precursors, but further discrimination from alternative cell lineages is required. Analysis of Abelson virus–transformed cell lines derived from fetal liver and bone marrow, combined with analysis of other lymphoma lines and normal tissues has established some of the molecular stages in the production of pre-B cells. The earliest event appears to be the rearrangement of the D_H and J_H regions observed in A-MuLV transformants of fetal liver cells as well as some T-cell lymphomas (9, 10, 11). This would not generate a functional mu heavy chain but requires further rearrangement to bring a variable region segment into place. The assembled V_H-D_H-J_H-C_μ allele can transcribe and translate a low level of mu protein which is carbohydrate modified and membrane bound but not usually exposed on the cell surface or secreted to interact with antigen (12, 13, 14, 15). Pre-B cells of this phenotype are first detected in low numbers in mid-gestational fetal liver and later in adult marrow where they can comprise up to 10% of the nucleated cells (7, 8).

A fraction of such pre-B cells undergoes rearrangement and expression of either a κ or λ light-chain gene in order to assemble complete IgM subunits as antigen-specific receptors on the cell surface. These surface IgM-positive B cells ($sIgM^+$) are infrequent in fetal liver until late in gestation but accumulate in the bone marrow and peripheral lymphoid tissues near parturition and following birth (16). Such $sIgM^+$ cells are probably capable of binding and responding to specific antigens. Further maturation after birth, including the transcriptional regulation of delta heavy-chain mRNA production and codeposition on the cell surface, produces $sIgM^+$-$sIgD^+$ cells (2, 17, 18). These cells are sensitive to antigen and mitogen activation and are found in adult spleen as the predominant resting B-cell population.

The final phase of B-cell maturation requires activation by an antigen, as well as helper factors from T and accessory cells, leading to both growth stimulation and induction of immunoglobulin secretion. This generally involves another major DNA rearrangement (class switching) to produce heavy chain isotypes other than μ (19, 20, 21); the use of alternative splicing choices to produce heavy chains with a secreted carboxy-terminal segment in large excess over the membrane-bound form (2, 22, 23); and cellular changes to generate the morphological and functional characteristics of the antibody-secreting factory of the plasma cell (6). We have roughly outlined the B-cell developmental pathway in Figure 1. Most of the various assay and culture systems we describe below are mentioned at that portion of the pathway that is best analyzed with each approach.

The discipline of immunochemistry provided the structural information and serological reagents to discriminate the different forms of secreted immunoglobulins. This enabled cellular immunologists to measure immunoglobulin production accurately as a functional correlate of B-cell maturation. Two different assay systems have been generally useful in

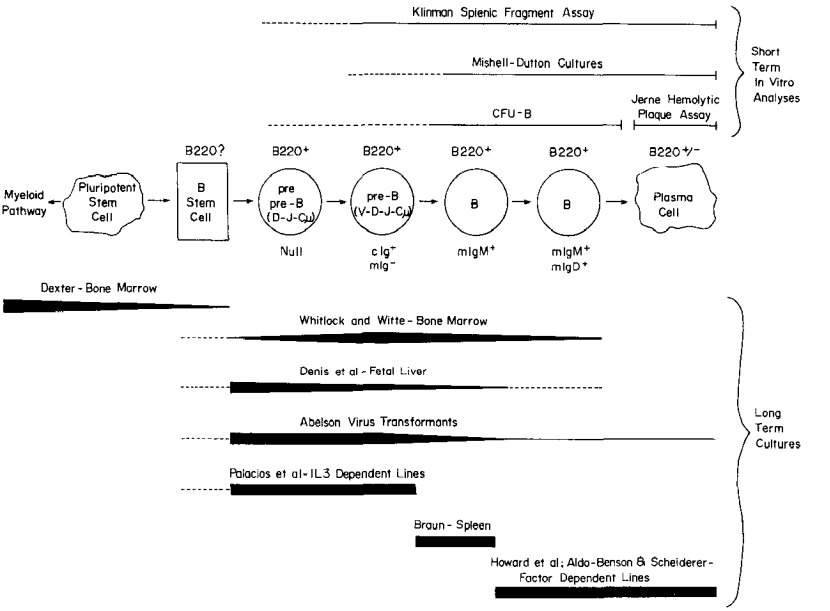

Figure 1 Scheme of B lymphocyte differentiation showing the stages of immunoglobulin gene rearrangement and expression and B220 antigen expression. The upper lines represent the B-cell stages monitored in a variety of short-term in vitro analyses. The lower lines represent the spectrum of cell types found in the long-term culture described in the text. The intensity of the line approximates the proportion of that cell stage in each culture type.

quantitating mature B-cell function. The Mischell-Dutton short-term culture system can be used to follow both B-cell proliferation (cell count, DNA precursor uptake) and immunoglobulin synthesis and secretion (24). Although generally designed for splenic B cells this method can be used for other lymphoid tissues to follow earlier steps in B-cell development (25, 26). Development of the hemolytic plaque assay by Jerne allowed enumeration of antibody-secreting cells (27). This quantification of immunoglobulin-secreting cells allows a relatively unambiguous assessment of antigen-specific responses (to RBC antigens or haptens) or, with appropriate modifications, of all immunoglobulin-secreting cells (28). Maturation of B cells can be followed and experimentally manipulated in vitro by the combination of these techniques. The role of lymphokines and other growth and differentiation factors can also be assessed in these systems (29). Advances in the development of serum-free media conditions have improved the sensitivity of such systems (30).

In short-term mass cultures the amount of proliferation monitored on any particular day of culture, or the number of antibody-secreting cells detected, is a function of both the number of functional precursors activated and the number of division cycles those activated cells undergo before analysis. Division often is not synchronous and varies between clones and in relation to different activating stimuli. Therefore it is difficult to extrapolate the data obtained from bulk cultures to determine the frequency of functional B cells or precursors in the original population. One approach to quantitating functional B cells or B-cell precursors is limiting dilution analysis (26, 31, 32). By culturing cells at decreasing densities and then using Poisson analysis of the number of responding cultures, the frequency of mature B cells responsive to specific antigens or mitogens in a heterogeneous population can be measured.

Monoclonal antigen-specific antibody responses of B lymphocytes have been obtained using the Klinman splenic fragment technique (33, 34, 35). In this limiting dilution system, lymphoid cells are injected into an irradiated, carrier-primed, syngeneic mouse. After the donor cells home to the recipient spleen, it is removed and divided into fragments. These splenic fragments are antigen stimulated in an in vitro culture using microwell plates, and then the antibody production is monitored. The use of irradiated and carrier-primed adult recipients provides excess T- and accessory-cell help and ensures that the B cells are maximally stimulated. In this manner, the frequency of response from a wide phenotypic range of B-lineage cells originating from numerous sources can be obtained using a spectrum of antigens (36).

A third method for quantitating B-lineage cells is the colony-forming B-cell assay (CFU-B). Membrane Ig-bearing B cells are capable of forming

soft agar colonies in response to mitogenic stimulation (37, 38, 39). The CFU-B assay detects B-lineage cells at a variety of maturation stages including IgD-positive and -negative stages and Ia-positive and -negative (7, 40, 41). However, pre-B cells that do not express membrane IgM are generally not capable of forming agar colonies unless allowed to differentiate to membrane Ig-bearing B cells either prior to soft agar culture or while cultured in the presence of feeder layers of fetal liver-adherent cells (41, 42, 43).

Early Phases of B-Cell Development

The functional assays discussed are useful for studying stimulation of resting, antigen-sensitive B cells to proliferate and differentiate to plasma cells. Other questions of interest are whether there are subpopulations of antigen-sensitive B cells with different functional capabilities, and what processes are involved in generation of the mature antigen-sensitive cell. A combination of approaches has helped in answering these questions. First was the characterization of the phenotypes and functional capabilities of B-lineage cells in various ages of fetal and neonatal tissues. Second was the development of assays and reagents for detecting cell-membrane proteins that are differentially expressed during B-cell development. And third has been the isolation and characterization of lymphoma cell lines that represent distinct stages of B-cell maturation. In addition to these approaches, a variety of in vitro assay systems have been developed in which immature populations of normal or transformed mouse B-lineage precursors can be driven to undergo limited differentiation.

During normal development of the fetus, functional B cells capable of responding to particular antigens and mitogens appear in a predictable order (44, 45). Responses to the mitogen dextran sulfate are detectable earlier than responses to bacterial lipopolysaccharide (LPS) (46, 47). Responses to thymus-dependent antigens and class 1 thymus-independent antigens also precede responses to class II antigens (47, 48, 49). Expression of membrane proteins such as Ia and IgD by spleen cells also follows a particular pathway. Both occur after birth of the animal, and Ia expression increases slightly before IgD expression (16, 18, 50). These and other membrane differentiation markers (CR, FcR, Ly-1, Qa-2, Mls, PC-1) have been used to distinguish B-cell subpopulations with different functional capabilities (49, 51–57). It is not clear whether mature B cells with different mitogen and antigen response profiles represent stages along a single linear maturation pathway or stages from two or more distinct branches of the main ontogenic pathway.

Little information existed on the characteristics of the maturation stages preceding expression of membrane Ig until lymphoma cell lines represent-

ing these stages become available. Cell lines isolated by transformation with A-MuLV have been useful in this regard. This defective retrovirus rapidly transforms pre-B cells in vivo or in vitro and has thus provided a means of generating numerous cell lines representative of this maturation stage (58–60). Although other cell types can serve as targets for transformation, the pre-B cell (with both heavy-chain genes rearranged and with light-chain genes unrearranged) is the preferred target (15, 61–63). Infection of fetal liver cells also results in transformation of pre–pre B cells that have only partially rearranged heavy-chain genes (9, 10).

Another lymphoma cell line that has been used extensively to study B-cell development is the 70Z/3 cell line (5, 64–65). This cell line synthesizes μ–heavy chain molecules and has a productively rearranged but unexpressed κ–light chain gene (66). 70Z/3 represents a maturational stage between the classical pre-B stage, in which light-chain genes are unrearranged, and the immature B-cell stage in which light chains are synthesized and membrane Ig is expressed. The importance of this cell line is that it turns on light-chain synthesis in response to treatment with stimuli such as LPS which have been shown to stimulate differentiation of normal pre-B and B cells (5, 64, 65).

Analysis of the early stages of B-cell ontogeny was aided by characterization of the B220 membrane protein which is a B lineage–specific protein expressed by all known stages of B-cell development except the plasma cell stage, at which expression is variable (67, 68). B220 is molecularly related to the T200 protein synthesized by T-lineage cells and may be related to a family of molecules that are expressed by most hematopoietic cells (69). A number of monclonal antibody reagents are available that recognize determinants on B220. Many of these reagents also weakly recognize a molecule expressed on a subpopulation of Ly-1^-, Lyt-2^+ cells which is a small percentage of normal mouse spleen. They all recognize the 220 kd protein on the membranes of the predominant T-cell population found in spleens of MLR-1pr/1pr mice (57, 67, 68, 70). One monoclonal, RA3-6B2, appears to be the most specific for the B-lineage B220 molecule (67). Other monoclonal antibodies that have been shown to recognize molecules on B-cell precursors are AA4.1 and GF1.2 (71). These antibodies provide a tool for isolation of precursors that can be used to study early B-cell maturation.

A number of approaches are now available for studying maturation of immature B cells in vitro. In addition to the 70Z/3 cell line, rare pre–B and pre–pre B A-MuLV-transformed cell lines have been found that will undergo limited maturation in vitro (9, 10, 72–78). These cell lines promise to provide an approach of unravelling the complex process of gene rearrangement and control of transcription and, hopefully, will give us

some clues as to which differentiation steps are preprogrammed and which, if any, are regulated by exogenous factors.

Differentiation of normal B-cell precursors from fetal tissues or adult bone marrow has been accomplished in vitro. The complexity of the culture conditions used to stimulate differentiation in each case suggests that the microenvironment plays a role in directing early B-cell maturation. Hammerling & Hoffman demonstrated that B-lineage populations enriched or depleted for cells expressing certain membrane antigens (Ia, Ig, CR, and PC-1) could be driven to express markers of the subsequent stage of maturation by treatment with LPS or agents that increased intracellular cAMP concentrations (50, 79–81). This technique was useful for delineating expression of these membrane proteins during B-cell development. Owen et al (13, 82) demonstrated that explanted fragments of day 12–16 fetal liver, spleen, or bone marrow provided the required microenvironment for murine B-lymphocyte development. These tissue fragments contained no detectable membrane Ig–bearing cells when first placed into culture but developed these cells after several days in vitro.

Similar studies were performed using short-term cultures of dispersed cell populations from fetal tissues. B-cell precursors were demonstrated in day 9–13 placenta (83) and embryonic blood (84) as well as day 12–19 fetal liver (25, 26, 85–87). The cultured cells were monitored by responsiveness to the mitogen LPS and subsequent development of antibody-secreting, plaque-forming cells. The cells from these various tissues were not immediately responsive to LPS but developed this responsiveness after several days in vitro. The peak of this response corresponded approximately to the in vitro equivalent of the day of birth.

Organ culture of fetal liver and fetal spleen was also used to demonstrate the presence of antigen-specific B-cell precursors. After several days in vitro the organ-cultured cells were stimulated with the hapten dinitrophenyl (DNP) coupled to a carrier protein in a Klinman splenic fragment assay (88). DNP-specific precursors could be detected as early as day 14 in fetal liver.

The CFU-B assay is another method for detecting pre–B-cell maturation. Only membrane Ig–bearing B cells are generally capable of forming agar colonies. Fetal liver or bone marrow pre-B cells will form colonies after short-term liquid culture under conditions that permit their differentiation to membrane Ig expression (41). Pre-B cells from bone marrow or fetal liver will differentiate to CFU-B if cultured in the proper microenvironment (41–43).

The development of B lymphocytes in vitro from fetal tissues that initially do not contain recognizable B cells has been taken as evidence that these

tissues possess the proper microenvironment for the commitment and differentiation of stem cells to B-lineage cells. However, due to the short culture intervals used in these studies, it is possible that only the later stages of development of fully committed pre-B lymphocytes were being monitored. Thus, it is not certain what sites, if any, are necessary for commitment of stem cells to B-lineage differentiation. The fetal liver and the adult bone marrow remain the most likely tissues for this commitment to occur.

The development of B lymphocytes from precursor cells occurs for only several days during gestation in the fetal liver and for the remainder of life in the bone marrow. It is possible that the precursor population and the events that lead to B-cell development in the bone marrow are fundamentally different from the events in the fetal liver. These two cell populations differ in a number of phenotypic and functional ways (7, 89), and it appears fetal liver–B lymphocytes are a less mature population than that found in bone marrow. It is not certain if either fetal liver or bone marrow alone can comprise a suitable microenvironment for the total spectrum of B-lymphocyte differentiation.

Long-term Growth Assays

Long-term maintenance of B-lineage cells in culture was needed in order to dissect out the components of B-cell development and to do further analysis of the phenotypes of immature B cells and the factors involved in directing differentiation. Analysis of normal cells freshly isolated from the animals is complicated by the heterogeneity of cell types present in hematopoietic tissues, especially bone marrow. Enrichment procedures always leave low numbers of T cells, macrophages, and other cell types that can make interpretation of assay results difficult.

An ideal culture system would allow long-term maintenance of normal B-lineage cell proliferation and would permit preparation of more homogeneous cell populations that still retain functional capabilities. This would permit examination of the cell types capable of extensive proliferation and of commitment to B-lineage differentiation, and would allow identification of the microenvironmental factors needed to promote these processes. With these goals in mind, many groups set out to design in vitro culture systems for extended growth and maturation of B-lineage cells. Two general approaches have proven successful. One is to add exogenous growth factors to promote growth. The second is to culture spleen cells or bone marrow cells for extended periods, after which continuous proliferation of lymphoid cells is established on adherent stromal cells.

The first reports came from Howard et al (90), who showed that splenic B cells would be cultured for an extended period in media containing factors synthesized by Con A–stimulated spleen cells. Cell lines specific for the

hapten DNP have been maintained in culture several months using a similar protocol (91). A recent approach using factors to maintain B-cell growth was reported by Palacios et al (92, 93). They showed that WEHI 3–conditioned medium (containing the growth factor IL-3) could be used to establish pre-B cell lines from bone marrow and spleen cells of various mouse strains. These cell lines are dependent upon IL-3 for proliferation. They do not proliferate in the presence of IL-1, IL-2, or gamma-interferon. All express AA4.1, and GF1.2 B-lineage membrane proteins, and most have rearranged heavy-chain genes and unrearranged κ light-chain genes. A fraction of the cells can develop in vitro to secrete immunoglobulin as measured in a hemolytic plaque assay system. Such lines should prove useful in evaluating factors promoting early and late stages of B-cell differentiation.

The second approach for establishment of long-term B-lineage cultures was to culture mixed cell populations in the absence of predefined factors. We reported that murine bone marrow cultures maintained in fetal calf serum at 37°C would after a period of several weeks establish continuously proliferating B, pre-B cell, and precursor populations that were dependent on an adherent stromal cell layer (94). The lymphoid cell fraction of such cultures was shown to contain not only mature membrane IgM–bearing cells, but also a large percentage of pre-B cells as well. Recently, Braun (95) reported that spleen cell cultures maintained for several weeks went through a similar process, with eventual establishment of B-cell lines that were consistently IgM^+, IgD^-, and Ly-1^+ and expressed λ-light chains. This B-cell subpopulation is a small fraction of the B-cell population in normal adult mouse spleen cells. In this culture system the B-lineage cell lines obtained do not appear to require adherent cells for maintenance of growth.

Long-term Bone Marrow Cultures

The bone marrow culture system we described provides a good opportunity for examining the early stages of B-cell development. Our primary goals were to characterize the growth and phenotypic properties of cells in the cultures, define the differentiative capacity of the cells, and to determine whether B-stem cells are maintained and expanded in vitro.

Basics of the Long-term Marrow Culture System

The conditions for establishment of long-term bone marrow cultures are similar to those used for short-term analysis of B-lymphocyte responses to antigens and mitogens. Bone marrow cells are cultured at 37°C in RPMI-1640 medium containing low concentrations of fetal calf serum (5%) and 5×10^{-5} M 2-mercaptoethanol (94). Cultures are initiated with 10^6 cells

per ml and no recharging with fresh bone marrow cells is necessary. A detailed description of the culture technique is published elsewhere (96).

These culture conditions differ from those employed by Dexter & Lajtha (97) to establish long-term bone marrow cultures useful for studying granulopoiesis. Dexter employed a DMEM-based media containing high concentrations of horse serum and maintained the cultures at 33°C. Corticosteroids have been shown to enhance growth in Dexter cultures greatly, and they may also be an essential component provided by selected batches of horse serum (98). The differences in the two culture methods result in a different distribution of adherent cell types in the stromal cell layers and the generation of very different microenvironments. In the Dexter culture system, the adherent stromal layer is made up of endothelial-like cells, dendritic-reticular cells, phagocytic mononuclear cells and lipid-filled adipocytes (99). No pre-B or mature B cells have been detected in Dexter-type cultures, but production of CFU-S and developing granulocytes is maintained for several months (100–103). Dexter cultures established in fetal calf serum often do not support the growth of CFU-S. The adherent cell population does not appear to contain the lipid-containing adipocytes (104). Granulopoiesis can be restored by the addition of hydrocortisone. Concomitant with the restoration of function is the appearance of the adipocytes (105). This strongly suggests that the adipocyte-like cells may be needed for the maintenance of CFU-S activity and the other nonlymphoid hematopoietic cells in the culture. Long-term B-cell cultures established as we describe provide an environment in which granulopoiesis ceases within 3–5 weeks, and B-lineage cells and their precursors continue to proliferate.

Phases of Culture Growth in Long-term B-Lineage Cultures

The phases through which the cultures progress are distinctive but vary in length according to the batch of fetal calf serum used to establish the cultures. In our original studies (94), the cultures required 5–8 weeks for lymphoid proliferation to begin. During the first two weeks adherent cells attached and proliferated to cover the bottom of the culture dishes, and the numbers of nonadherent cells rapidly declined. After three weeks of culture few viable, nonadherent cells could be detected in the cultures, and little change was seen for the next 1–3 weeks. This phase during which few nonadherent cells were seen was termed the crisis phase because it resembled what Todaro & Green (106) described for primary cultures of mouse-embryo fibroblast cells. After the crisis phase, patches of nonadherent cells reappeared on the adherent cell layer, and these rapidly expanded in size until the cell numbers in the cultures reached a stable plateau of about $1–2 \times 10^5$ cells per ml.

We currently choose batches of fetal calf serum that routinely give rise to B-lineage cell lines in all cultures within 3–4 weeks. As a result of this shorter time for establishment, little discrete crisis phase is seen. Often lymphocyte proliferation can be seen in the cultures as early as 1–2 weeks after initiation. Granulocyte proliferation still ceases after 3–4 weeks.

Turnover of Lymphoid Cells in the Cultures

The nonadherent cell number in established cultures is relatively stable if the cultures remain untouched except when the culture medium is changed. If 90% of the nonadherent cells are removed from the cultures, the original cell numbers are reestablished in about 3–5 days, and again remain stable until more cells are removed. Recent analysis by autoradiography of ^{3}H-thymidine-labeled cells indicated that a large proportion of the cells from cultures that were not expanding in total cell number were actively dividing (C. Whitlock, unpublished data). As many as 25–45% of the nonadherent cells incorporated ^{3}H-thymidine during a four hour labeling period. At a certain cell density, there is a balance between division and cell death occurring in the cultures, and somehow this turnover of cells is slowed when the cell number is decreased. How turnover of cells is controlled in the cultures may relate to how the size of the B-cell compartment is regulated in the animal.

Phenotypes of the Cultured B-Lineage Cells

The first indication that B cells were among the small, nonadherent cells proliferating in the cultures came from biosynthetic labeling experiments which showed a large amount of Ig–heavy and –light chain synthesis (94). We have characterized the phenotypes expressed in numerous cultures in order to establish the range of B-cell stages that can be supported by this culture system. Under phase microscopy, the cells resemble small lymphocytes. They vary in size over a small range, probably because they are cycling, but rarely are cells seen that are the size of the blast cells observed in mitogen-stimulated spleen cell cultures.

The percentage of nonadherent cells in the cultures that express membrane IgM, IgD and Ia varies between individual cultures. When individual cultures are analyzed at four weeks after initiation, one observes cultures that have few membrane Ig-bearing cells (10 to 15%) and others have nearly 100% membrane Ig^{+} cells (C. Muller, unpublished data). Most cultures contain 10–20% membrane Ig–bearing cells in the first three months of culture, and these B cells synthesize a heterogeneous (polyclonal) population of light- and heavy-chain molecules (94). Initially, very little IgD is found expressed, but expression of this isotype increases as the cultures are maintained for several weeks (96). At 15–20 weeks, subpopulations of

either pre-B or B cells tend to dominate the cultures, and the pattern of heavy- and light-chain molecules synthesized becomes pauciclonal (94). The distribution of cells bearing membrane-Ig at this point tends to be either 0% or 100%, and those cells expressing membrane IgM generally express IgD. Preliminary studies show that these cells also express Ia molecules (C. Whitlock, unpublished data).

Characterization of the Null Cell Population

The proportion of nonadherent cells that do not express membrane-Ig is 80–90% of most cultures early after establishment. By Wright's staining of cytocentrifuge preparations, all of the nonadherent cells appear to be lymphocytes and none express Thy-1 antigens. With immunofluorescent staining with RA3-6B2 (anti-B220), most of the null cell population can be shown to belong to the B lineage, but 10–30% have very low to undetectable amounts of this protein (C. Whitlock, J. Kurland, unpublished data).

The amount of B220 on B-lineage cells from the animal as detected by immunofluorescence is less on bone marrow cells than spleen cells (C. Whitlock, unpublished observation). This suggests that immature and mature B-lineage cells may be segregated by using fluorescence-activated cell sorting. In one experiment, cells from young cultures were pooled, stained with RA3-6B2, and the 25% of the cells with the lowest and highest densities of B220 were collected on the cell sorter. Each population was metabolically labeled with ^{35}S-methionine, and cell lysates were immunoprecipitated with rabbit antimouse-Ig and rabbit antiterminal deoxynucleotidyl transferase (TdT). TdT is a nuclear protein synthesized by immature T cells in the thymus and pre-B cells (107–110). The cells with low densities of B220 synthesized low amounts of mu immunoglobulin and high amounts of TdT (C. Whitlock, unpublished data). Cells with high B220 densities exhibited the reverse pattern—low amounts of TdT and easily detectable amounts of mu heavy chains. This indicates that one cell type belonging to the null population is a cell that expresses TdT but synthesizes few or no mu–heavy chain molecules. Furthermore, these cells can be enriched by using fluorescence-activated cell sorting to segregate cells with different B220 staining patterns.

DNA extracted from the nonadherent cells of these cultures has been analyzed for rearrangements at the immunoglobulin heavy chain locus by Southern blots (111). In parallel to the heterogeneity of immunoglobulin protein expression, young cultures have numerous rearranged mu-gene bands while older cultures (>3 mo.) have several predominant bands. In most cultures examined, however, approximately 10–30% of the heavy chain loci were in the unrearranged germline state. It is possible that the

cells with unrearranged immunoglobulin–heavy chain loci represent very early B-lineage cells. No method currently exists for enrichment of such a population from the highly heterogeneous hematopoietic tissues where these cells reside in the animal. The isolation of these cells from long-term cultures would be useful for characterization of this cell type.

Further evidence for the presence of a B-cell precursor subpopulation in long-term bone marrow cultures was obtained by successful reconstitution of the B-lymphocyte compartment in genetically defective mice (112). (CBA/N × BALB/c)F_1 male mice, which lack CFU-B cells and TI–, TII–responsive B cells, were injected with cells from long-term bone marrow cultures. These cultured cells were capable of restoring the defective humoral immunity in these mice. This suggested that a B-lymphocyte progenitor which could be maintained for long periods in vitro was able to repopulate at least a portion of the B-lymphocyte compartment in vivo. The kinetics of this reconstitution were compatible with the expansion and differentiation of such a progenitor.

Long-term cultured B-lineage cells have also been used for short-term repopulation exclusively of the B-lymphoid compartment of severe combined–immune deficiency mice (113, 114). In contrast, both the T- and B-cell lineages of these mice can be reconstituted with either normal bone marrow (115) or Dexter-type cultured bone marrow cells (114). Further evidence for functional B-cell precursors in the long-term B-cell cultures comes from a report by Nagasawa et al (116). They showed that cultured pre-B cell populations could develop in vivo to LPS-responsive cells.

Use of Long-term B-Cell Cultures to Study Differentiation

Two immediate goals were to obtain clones of B-lineage cells useful for biochemical analysis and to determine the maturational and functional capacities of the cultured cells. We assumed from the high percentage of null cells in the cultures and the heterogeneity of Ig molecules synthesized by the B cells that differentiation from early precursor cells was occurring. Established cultures were cloned by limiting dilution culture on adherent cell layers in 96-well culture plates (76). We knew that cloning would favor growth of those cells with high proliferative capacity, and we hoped this might enhance our chances of isolating very early B-cell precursors and possibly B-stem cells. Analysis of the cloned populations showed that most of the lines were pre-B and B cells and that in each cloning most of the lines were siblings derived from a single precursor cell with a specific pattern of heavy chain–gene rearrangements (76). Although the lines were related, they exhibited a variable pattern of unrearranged and rearranged kappa chain genes, and they synthesized either no light chains or light chains that

were discernible on two-dimensional polyacrylamide gels. These results confirmed that differentiation of pre-B cells to B cells was occurring in the cultures.

We have been able to study differentiation of some of the pre-B cell clones indirectly by using transformation with A-MuLV to analyze the progeny of single cells (117). After infection of the cultured cells and soft agar culture, the agar colonies were analyzed for immunoglobulin–heavy and –light chain synthesis. In several cases the cells from a single colony were found to have synthesized a wide variety of light-chain molecules. Analysis of subclones from these populations showed that each cell line that synthesized a unique kappa protein molecule (as determined by migration on two-dimensional polyacrylamide gels) also had a unique kappa light-chain gene rearrangement. The subclones of one particular transformed pre-B cell all expressed kappa chains with a single, variable-region type (V_k21B). Interestingly, three different protein species separable on two-dimensional gels could be identified. Restriction-blot and partial-sequence analysis has demonstrated that somatic mutation of the germline V_k21B segment has occurred in two of the three kappa genes (117; S. Ziegler and O. Witte, unpublished). These alterations indicate that somatic mutation of rearranged light-chain genes can occur in the absence of class switching.

Analysis of another cloned cell line was instrumental in showing that very early B-lineage cells existed in the cultures that had the potential for differentiation. When this slowly growing line was transformed with A-MuLV, and the agar colonies analyzed, we found them each to have different patterns of heavy-chain gene rearrangements (76). Three had kappa light-chain gene rearrangements, but none synthesized detectable light-chain molecules. When enough cells were collected from the original cloned cell line for DNA analysis, the dominant heavy-chain gene rearrangements found were a subpopulation of the rearrangements seen for the transformants. We concluded from these data that the original cloned population resulted from a very early cell at or prior to the stage where heavy-chain genes are rearranged and that rearrangement of heavy-chain genes occurred in the progeny of this precursor after cloning. Further evidence that this population derived from a very early cell type is that only one of the transformed subpopulations synthesized heavy-chain molecules. Those transformed subclones that fail to synthesize mu heavy chains had rearrangements that were similar in size to the predominant rearrangements found in A-MuLV-transformed pre–pre B cells obtained from fetal liver cell infections (9, 10; S. Ziegler, O. Witte, unpublished data). This suggests that these may represent nonproductive D_h-J_h rearrangements which are an intermediate step toward productive heavy-chain gene rearrangement.

Fetal Liver Cultures

The small numbers of B-lineage cells present in fetal tissue for a short period of time make these cells difficult to study. Fundamental differences between B cells and their precursors from fetal liver and bone marrow have been described, but short-term culture methods are unable to discriminate between a temporal effect or true inherent differences between the two compartments. Further study of the normal differentiation pathways of B-lineage cells from fetal liver and comparison of these to B-lineage cells from bone marrow, required development of long-term culture methods for fetal liver cells. B-cell cultures have been established from fetal liver using a modification of the conditions used for bone marrow (94, 111). These fetal liver cells are dependent for their long-term growth upon a feeder layer of adherent bone marrow cells. The adherent layer formed by fetal liver cells is able to support only short-term growth of B-lineage cells from fetal liver. It is possible that stromal elements from fetal liver comparable to those from bone marrow, do not exist or are unable to survive in culture. Long-term fetal liver cultures are established by plating a single cell suspension of fetal liver cells upon bone marrow–derived adherent layers. Transfer of the nonadherent fetal liver cells to a second bone marrow–derived adherent layer after 7–10 days of culture is also crucial. It is not known whether this is due to an inhibitory or toxic cell present in fetal liver or to other causes.

The fetal liver cultures appear morphologically similar to the long-term bone marrow cultures. The nonadherent cells are relatively homogeneous in size and grow in patches upon the adherent layer of cells. These nonadherent cells are lymphoid in morphology when stained with Wright-Giemsa. Cytoplasmic staining for immunoglobulin μ heavy chain is also similar for fetal-liver and bone marrow cultures ranging from 15–50% of the cells (111). In contrast, mIg^+ cells, which comprise approximately 10–15% of 2–3 month old bone marrow cultures are less than 1% of the cells in fetal liver cultures of similar age. Thus, the fetal liver cells, although cultured upon bone marrow stromal cell–adherent feeder layers, appear to represent a less mature B-lymphocyte population.

Southern blot analysis of immunoglobulin–heavy-chain genes from the long-term cultured fetal liver cells shows approximately 70–90% of the heavy chain to be rearranged at the J_H locus (11). Therefore, most of the cultured fetal liver cells that have a null immunoglobulin phenotype are of the pre–pre B cell type with rearranged but unexpressed immunoglobulin–heavy chain genes. It is important to note that after months in culture a subpopulation of the cultured fetal liver cells retains germ line–configuration immunoglobulin genes on at least one chromosome. These fetal liver cultures, as in the bone marrow cultures, may contain cells very early

in the B-cell lineage which are capable of undergoing immunoglobulin gene rearrangement. These presumptive B-lymphocyte progenitors may be responsible for the generation of the more mature members of this lineage in long-term culture.

The early B-lineage cells in the fetal-liver culture can also serve as targets for A-MuLV transformation (111). Cloned transformants of cultured fetal-liver cells are mainly of a null immunoglobulin phenotype with only 19% synthesizing mu heavy chain. No kappa synthesis has been observed in these cultured fetal liver transformants. This distribution of phenotypes is very similar to that obtained when fresh fetal liver is transformed with A-MuLV (10). In contrast, cloned transformants of cultured bone marrow cells are approximately 50% mu^+ with a small percentage of mu^+ $kappa^+$ transformants (76, 94). This is similar to the distribution of phenotypes obtained after A-MuLV transformation of fresh bone marrow (10). The cultured fetal liver cells again appear to be a less mature B-lineage population than the cultured bone marrow cells by this analysis.

Role of the Adherent Stromal Cells in B-Lineage Cell Proliferation In Vitro

Ultrastructural and light microscopy analysis of the bone marrow shows that the hematopoietic cells share a consistent spatial relationship with the stromal microenvironment. In the developing chick bone marrow, erythroid and granulopoietic precursor cells segregate into specific regions in the bone marrow. This segregation is controlled by the stromal microenvironment. The erythroblast associates with sinusoidal endothelium through an extensive array of microtubules while the granulopoietic cells establish a less extensive association with the extravascular reticular cells (118). Other electron microscope analysis of erythroid islands in the rat have shown that macrophages appear to be necessary for reestablishment of erythropoiesis after induced erythroid depression (119). These and other lines of evidence (120, 121) suggest that there is a required interaction between hematopoietic cells and the stromal environment in vivo.

The cells in the stroma may simply provide a supportive matrix on which hematopoiesis occurs, or they may play some regulatory role in development. A variety of in vitro assays show that diffusable factors play a significant role in the proliferation and development of hematopoietic-lymphoid cell lineages (42, 122). The importance of the microenvironment is also shown directly by the genetic defect of the Steel (S1) mice (123). Compared to normal mice, these mice have half the number of hematopoietic stem cells (CFU-S). The erythropoietic defect can be "cured" in vivo by implanting a normal splenic environment, therefore restoring active erythropoiesis (124). Further experiments in vitro have shown that

erythropoiesis from S1 marrow can be restored in the presence of an adherent cell layer from normal mouse bone marrow (125). These results show that CFU-S activity can be restored in an S1 mouse with a normal adherent microenvironment.

In the long-term B-cell cultures prepared as described by Whitlock & Witte (94), the adherent cell population consists of a mixed population of fibroblasts, reticular-dendritic cells, epithelial-like cells, and mononuclear cells. The cells grow in large clusters which cover 70–80% of the plate. The lipid-filled adipocytes characteristically found in the Dexter culture are noticeably absent from these B-cell cultures. The mass population of B cells and the B-cell clonal lines which grow in our culture system are directly dependent on this adherent stromal layer for growth and viability. Light microscopic analysis of these cultures shows that the nonadherent B cells grow as foci on or near specific cell types in the adherent stromal layer. This growth pattern creates the cobblestone effect previously described by Dexter (99).

The long-term growth of B cells in vitro is supported by a soluble factor that is secreted by the adherent stromal layer (D. Robertson, O. Witte, manuscript in preparation). This activity can be measured by following the uptake of ^{3}H-thymidine by the mass population of cultured B cells or the established clonal lines in the presence of the culture supernatant.

Efforts were made directly to isolate this cell type from the adherent cell population and to establish a continuous cell line which could support B-cell growth. Nine single-cell clones were eventually obtained after cells were plated using limiting-dilution cloning. The clonal lines consist of mononuclear cells with a large cytoplasm, as shown in a Wright-Giemsa stain. The cells have long extensions which often interconnect. They do not phagocytose latex beads or pinocytose neutral red as compared with macrophages or macrophage-derived cell lines. In addition, the cytoplasm does not stain for macrophage-specific lipase. These cloned cell lines are probably distinct from reticular-dendritic cells found in the uncloned population since they do not express detectable Ia antigens on their surface. Other histochemical analyses show that the cells do not stain positively with sudan black or Schiff's reagent suggesting that the cells are not in the granulocytic lineage (D. Robertson, O.Witte, manuscript in preparation).

These cloned adherent cell lines have provided a useful system to study the nature of the secreted–growth promoting activity. Once established these clonal cell lines can be maintained in serum-free media for several days. The growth factor(s) is a secreted, trypsin-sensitive, heat-stable protein. The target cell of this growth factor appears to be restricted to lymphocytes early in the B-cell lineage. Unlike other lymphokine growth factors, this bone marrow–derived factor does not stimulate the prolifera-

tion of LPS-activated splenic B cells, B cells derived from the BCL-1 tumor, or IL2- and IL3-dependent cell lines (D. Robertson, unpublished observations).

This in vitro culture system has been established in an attempt to create an in vitro environment which closely mimics the environment found in the lymphoid organ. The dissection of this stromal : lymphoid cell interaction has been simplified by the establishment of clonal cell lines which support the growth of long-term B lymphocytes. We are not convinced that the presence of a single growth promoting activity is completely responsible for the proliferation of the lineage cells in the in vitro culture system. Supernatants conditioned by the adherent layer do not support the short- and long-term growth of the B cells with the same growth kinetics as those cells growing in the presence of an uncloned adherent layer. This slower growth rate and slightly reduced viability indicates that an additional factor may be involved in the control of lymphoid proliferation.

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CONTENTS

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T-LYMPHOCYTE RECOGNITION OF ANTIGEN IN ASSOCIATION WITH GENE PRODUCTS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX[1]

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INTRODUCTION

One of the most important conceptual breakthroughs in cellular immunology during the 1970s was the realization that the influence of gene products of the major histocompatibility complex (MHC) on immune reactions stemmed largely from the central role they played in the activation of T lymphocytes (1). Experiments with both cytotoxic and inducer lymphocytes demonstrated that the T cells had to co-recognize antigen in association with one of these MHC-encoded molecules in order for activation to occur. Cytotoxic T cells required class I molecules whereas inducer T cells required class II molecules.

Thus, in contrast to other cell-surface receptors specific for a single ligand (e.g. a hormone receptor) the antigen-specific receptor on T cells must form a ternary complex with two ligands. One of these ligands is a transmembrane glycoprotein on the surface of another cell; the other is often an unknown partial degradation product of the original antigen added to the cultures. As a consequence of this complexity, no simple ligand binding assays exist. All receptor interactions are measured with a biological assay, such as thymidine incorporation, which appears to reflect in a quantitative

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way the events that initiate the response (2), presumably because the first steps are rate limiting.

Partly because of its complexity and partly because of its biological importance, the molecular basis of the recognition event has intrigued immunologists for a long time and has been the central focus of research in a number of laboratories during the 1980s. The recent advances in this area have come on two fronts. First, the genes have been cloned that encode histocompatibility molecules (3) and the antigen-specific T-cell receptor (4–6), and simple structural models of the proteins have been proposed based on sequence homology with immunoglobulin (7). Second, a number of functional experiments have suggested that the histocompatibility molecule and the antigen physically interact during the T-cell activation event (8–11). In this review I attempt to summarize these recent structural and functional experiments and then to synthesize them into a few models that have become the major viable alternatives for explaining the phenomenon of MHC-restricted recognition of antigen by T cells. In addition, I present a few new speculations on the evolutionary explanation for this type of recognition.

STRUCTURAL ORGANIZATION OF THE T-CELL RECEPTOR THAT RECOGNIZES ANTIGEN IN ASSOCIATION WITH A HISTOCOMPATIBILITY MOLECULE

Biochemical and Molecular Information

The introduction of T-cell cloning technology opened the way to the isolation of the T cell antigen-specific receptor (12). Homogeneous populations of cells were used to immunize and characterize antibodies against the receptor, and these antibodies in turn were used to isolate the molecule (13–16). Several groups (17) have reported that the receptor is a heterodimer composed of two polypeptide chains linked together by interchain disulfide bond(s). The two chains can be distinguished on the basis of pI in isoelectric focusing gels (the acidic chain has been designated α, the neutral to slightly basic chain, β) as well as by differences in peptide maps. Each chain is a transmembrane glycoprotein with a core size of approximately 32,000 daltons that is composed of clonotypically variable and constant peptides. From the nucleotide sequence of the β chain (18) it is clear that the protein is organized into one variable- and one constant-region domain, each of which contains an intrachain disulfide bond and possibly an interdomain disulfide bond (19).

At the DNA level the β genes have been found to be fragmented into V, D, J, and C components, which rearrange during differentiation to yield the

expressed gene (18). In some cases V can join directly to J. In other cases there is evidence for the participation of more than one D segment. These rearrangements could give rise to a large degree of diversity through combinatorial joining of different V, D, and J genes. Interestingly, the first DNA encoding the variable region (V_T) to be sequenced at both the cDNA and germline level demonstrated that the expressed V_T gene was identical to the germline gene (18). Whether this means somatic hypermutation will not play a significant role in T cell–receptor diversity remains to be determined from more extensive sequence analysis.

Overall, the structure of the T-cell receptor appears to be very similar to that of immunoglobulin. There is significant amino acid homology between the two. In both the receptor and in immunoglobulin heavy chains a D segment is present in the V region and transmembrane and cytoplasmic segments occur in the constant region. Even the region around the connecting interchain disulfide bond is encoded as a separate exon and shows homology to the immunoglobulin hinge region (7, 18). On the other hand, the genomic organization of the β chain gene is similar to that of the lambda light chain, with two sets of J and C genes. Thus, the T-cell receptor is clearly a unique amalgam of the previously defined properties of different immunoglobulins. Nonetheless, it seems reasonable to postulate that the T-cell receptor is composed of two transmembrane immunoglobulin-chain analogs linked together by disulfide bond(s). The critical question for protein-molecular model building is whether the two V regions interact with each other, like heavy and light chains, to form a single combining site for both antigen and the Ia molecule, or whether the two V regions are independent of one another, as two heavy chains are in immunoglobulin, and thus free to interact separately with antigen and the Ia molecule.

Functional Information

Early studies on cytotoxic T cells demonstrated that the antigen and the histocompatibility molecule had to be presented at the same time on the same target cell in order for lysis to occur (20). However, the relationship between the antigen-specific and MHC-specific recognition components of the T-cell receptor remained unexplored until the advent of T-cell cloning. In 1981 Kappler et al (21) were able to fuse two T cells of different specificities and ask whether the antigen- and MHC-specific components could segregate independently. A drug-marked T-cell hybridoma specific for ovalbumin (OVA) in association with the $A^k_\beta : A^k_\alpha$ Ia molecule was fused to a normal T-cell line specific for keyhole limpet hemocyanin (KLH) in association with the $A^f_\beta : A^f_\alpha$ Ia molecule. The resulting cloned somatic hybrid could be stimulated to release IL-2 by either of the original pairs of antigen and Ia molecule, but not by OVA in association with $A^f_\beta : A^f_\alpha$ or KLH in association with $A^k_\beta : A^k_\alpha$. Similar results were obtained in 19 other

hybrids, although rigorous proof of clonality was lacking in those cases. The authors concluded from these experiments that the recognition of antigen was not independent from the recognition of the Ia molecule.

The only data in the literature that contradicts these observations is in a paper by Lonai et al (22). T cells specific for nitrophenylated chicken gammaglobulin (NP-CGG) and the $A^b_\beta : A^b_\alpha$ Ia molecule were fused with BW-5147, an $H\text{-}2^k$-bearing thymoma. Many of the resulting hybrids could be stimulated by NP-CGG in association with presenting cells bearing $H\text{-}2^k$ as well as presenting cells bearing $A^b_\beta : A^b_\alpha$. The authors suggested that the BW-5147 might have contributed a receptor chain specific for Ia^k and that the antigen-specific chain associated with both of the anti-MHC-specific chains to produce the dual specificity of the clone. Although a positive result of receptor scrambling would certainly be worth more than many negative results, there is one critical control lacking in this experiment. The T cells specific for NP-CGG and $A^b_\beta : A^b_\alpha$ were never assessed for reactivity with NP-CGG in association with Ia^k *before* the fusion with BW-5147. As will be discussed later, T-cell clones exist that are MHC-degenerate, i.e. are capable of recognizing antigen in association with more than one allelic form of Ia molecule. If this were the case for the NP-CGG clones, then scrambling of specificities is not the correct interpretation of the data.

Nonetheless, it is clear from the structural data that two receptor chains do exist (α and β) and that potentially they could scramble with another pair (α' and β'). The efficiency of hybrid formation (α and β' and β and α') would depend on whether association of all α and β chains is random. If there is preferential chain association, as is sometimes seen for heavy and light chains of immunoglobulin, then certain chain combinations might not lead to scrambling. The Kappler et al data suggest that chain scrambling to produce a functional hybrid specificity is rare ($< 1/20$). This means that the anti-MHC specificity and the anti-antigen specificity are either not segregated in a chain specific manner or, if they are segregated, that the Ia molecule and the antigen physically (or functionally) interact in such a way as to preclude a ternary complex from forming or from activating the T cell through a scrambled receptor (see below).

ANTIGEN PRESENTATION EXPERIMENTS

Evidence for Interaction of Ia Molecules and Antigen

T lymphocytes from B10.A mice immunized with pigeon cytochrome *c* in Freund's complete adjuvant have been shown to display a proliferative response in vitro when rechallenged with the antigen in the presence of syngeneic presenting cells (23). The specificity of the response, as defined by

stimulation of the cells with different species variants of cytochrome *c* and different chemical and synthetic fragments of the immunogen (Table 1), was highly reproducible; and the pattern indicated that a single dominant antigenic determinant existed at the carboxyl-terminal end of the molecule. In particular, moth cytochrome *c* and its C-terminal fragment, amino acids 81–103, always elicited a heteroclitic response, i.e. it was more potent on a molar basis than the immunogen, pigeon cytochrome *c*. In contrast to B10.A mice, many other B10 congenic strains, differing only in MHC haplotype, were nonresponders to pigeon cytochrome *c*. Some of these strains, such as B10.A(5R), could respond to some cytochromes *c* after immunization with the more potent moth cytochrome *c*; but a T cell–proliferative response to pigeon cytochrome *c* could still not be elicited (24). These results suggested that the T-cell repertoire for pigeon cytochrome *c* might be quite limited and prompted an exploration of the degree of receptor diversity at the clonal level.

Out of these studies emerged the first convincing experimental evidence that Ia molecules and antigen interact with one another during the T-cell activation process. Ellen Heber-Katz in my laboratory discovered that most of the B10.A ($E^k_\beta : E^k_\alpha$) T-cell hybridomas specific for pigeon cytochrome *c* could also be stimulated by moth cytochrome *c* in association with B10.A(5R) ($E^b_\beta : E^k_\alpha$) antigen-presenting cells (APCs) (8). No other APCs bearing different *H*-2 haplotypes, including APCs from B10 and B10.A(4R) mice (no $E_\beta : E_\alpha$ expression at the cell surface), gave any stimulation. Thus, the clones were capable of recognizing moth cytochrome *c* in association with either the $E^k_\beta : E^k_\alpha$ or the $E^b_\beta : E^k_\alpha$ Ia molecule. This selective degeneracy allowed us to examine the fine specificity of antigen recognition under conditions in which the T-cell receptor was kept constant and only the Ia molecule was varied. Such experiments demonstrated that the pattern of antigen reactivity changed when the Ia molecule was changed. For example, in the presence of B10.A APCs the T-cell clones could be stimulated by pigeon cytochrome *c*, but in the presence of B10.A(5R) APCs they could not.

One could argue that the antigen response with B10.A(5R) APCs represented a weak cross-reaction of an anti-Ia^k receptor with Ia^b molecules, and that because the cell sums signals from both its anti-antigen and anti-MHC receptors, only potent antigens such as moth cytochrome *c* would stimulate with B10.A(5R) APCs. Weaker analogs such as pigeon cytochrome *c* would not stimulate. However, testing a battery of different analogs it was possible to show some reversals in the rank order of stimulation when different Ia molecules were used (8). The most dramatic example of this (shown in Figure 1) came from a second series of clones described by Lou Matis and Steve Hedrick in the laboratory (25).

Table 1 Cyanogen bromide and *synthetic carboxyl-terminal* peptides of cytochrome *c* used to stimulate T cells

Antigen	Amino acid sequence[a]																						
Pigeon 81–104	I	F	A	G	I	K	K	K	A	E	R	A	D	L	I	A	Y	L	K	Q	A	T	A	K
Moth 81–103	V	—	—	—	L	—	—	A	N	—	—	—	—	—	—	—	—	—	—	—	—	—	K	
Des-Ala-pigeon 81–104	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K	
Acetimidyl-pigeon 81–104	—	—	—	—	—	B	B	B	—	—	—	—	—	—	—	—	—	—	B	—	—	—	—	B
Acetimidyl-moth 81–103	V	—	—	—	L	B	B	A	N	—	—	—	—	—	—	—	—	—	B	—	—	—	B	
Moth 88–103								A	N	—	—	—	—	—	—	—	—	—	—	—	—	—	K	
Acetimidyl-moth 88–103								A	N	—	—	—	—	—	—	—	—	—	B	—	—	—	B	
Tuna 81–103	—	—	—	—	—	—	—	—	G	—	—	Q	—	—	V	—	—	—	—	S	—	—	S	
Acetimidyl-tuna	—	—	—	—	—	B	B	B	G	—	—	Q	—	—	V	—	—	—	B	S	—	—	S	
Moth 86–90; 94–103									K	K	A	N	E	—	—	—	—	—	—	—	—	—	K	
Moth 86–90; 94–103(Q99)									K	K	A	N	E	—	—	—	—	—	Q	—	—	—	K	
Moth 86–90; pigeon 94–104									K	K	A	N	E	—	—	—	—	—	—	—	—	—	—	—
Moth 86–90; pigeon 94–104(Q99)									K	K	A	N	E	—	—	—	—	—	Q	—	—	—	—	—
Moth 94–103														—	—	—	—	—	—	—	—	—	K	
Moth 97–103																	—	—	—	—	—	—	K	
Moth 93–103(E93, Q99)													E	—	—	—	—	—	Q	—	—	—	K	

[a] Amino acids are designated by the single letter code corresponding to the first letter of their names with the exceptions of aspartic acid (D), asparagine (N), glutamine (Q), glutamic acid (E), lysine (K), *phenylalanine* (F), *tryptophan* (W), and tyrosine (Y). B indicates the acetimidyl-derivative of lysine described in the text.

Approximately 5–10% of B10.A clones specific for pigeon cytochrome *c* are not degenerate with B10.A(5R) APCs but can be stimulated with B10.S(9R) APCs ($E^s_\beta : E^k_\alpha$). These clones showed the typical pattern of moth cytochrome *c* fragment 81–103 being more potent than pigeon cytochrome *c* fragment 81–104 when B10.A APCs were used (Figure 1). However, when B10.S(9R) APCs were used the pattern reversed. The potency of moth cytochrome *c* fragment 81–103 decreased 100-fold while the potency of the pigeon fragment 81–104 increased 10-fold. This shift in opposite directions can not be explained by a "dual receptor-compensating affinity" model of T-cell activation. The data argue strongly that the Ia molecule influences either directly or indirectly the antigen specificity of the response.

The extent of the degeneracy of B10.A clones with B10.A(5R) APCs (90–95% of all clones) prompted us to investigate the degeneracy in the opposite direction (8, 24). Surprisingly, many B10.A(5R) clones elicited by immunization with moth cytochrome *c* 81–103 were selectively degenerate on B10.A APCs. In addition, the fine specificity of antigen recognition changed with the APC. The B10.A(5R) clones gave a "B10.A pattern" of responsiveness when B10.A APCs were used and a "B10.A(5R) pattern" when B10.A(5R) APCs were used. For example, the clones responded to pigeon cytochrome *c* fragment 81–104 with B10.A but not B10.A(5R) APCs. This phenomenon

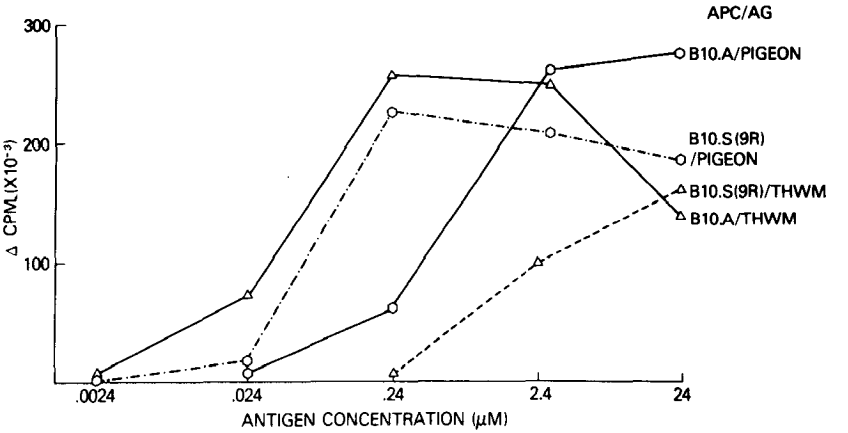

Figure 1 Switching allelic forms of the Ia molecule alters the antigenic fine specificity of T-cell activation. The normal T-cell clone, A3.G4, was derived from B10.A mice immunized with pigeon cytochrome *c* by establishing long-term lines in vitro and cloning by limiting dilution (25). This clone could be stimulated to proliferate (ΔCPM) with cytochrome *c* in association with either B10.A or B10.S(9R) antigen-presenting cells (APC). This figure shows how the antigenic potency of different cytochromes *c* changes with the different allelic forms of the Ia molecule. The four combinations shown are: B10.A APCs and pigeon cytochrome *c* (○—○); B10.A APCs and tobacco hornworm moth (THWM) cytochrome *c* (△—△); B10.S(9R) APCs and pigeon cytochrome *c* (○-·-○); B10.S(9R) APCs and THWM cytochrome *c* (△– – –△).

was also observed at the population level, where about half the maximum proliferative response of B10.A(5R) T cells to moth cytochrome *c* fragment 81–103 in association with B10.A(5R) APCs could be achieved with B10.A APCs. These results suggest that many of the cytochrome *c*–specific T-cell clones in the B10.A(5R) mouse have similar antigen and MHC-specific receptors to those on cytochrome *c*–specific T cell clones in the B10.A. The implications of this unusual observation for immune response (Ir) gene function have been discussed in detail elsewhere (24).

Recently this B10.A and B10.A(5R) degeneracy of T-cell clones has been observed with other antigens (26). B10.A T-cell hybridomas specific for PPD or the herpes virus D protein in association with the $E^k_\beta : E^k_\alpha$ Ia molecule were shown to respond to these antigens in association with $E^b_\beta : E^k_\alpha$. The generalization of this phenomenon to several antigens suggests that the explanation of the degeneracy at the molecular level is likely to lie with the Ia molecule. Mengle-Gaw & McDevitt (27) and Widera & Flavell (28) have recently cloned and sequenced the E^k_β and E^b_β genes, respectively. A comparison of the two sequences showed an interesting finding. The region around residues 68–75 in the first domain (β_1), which is normally quite variable in sequence between other E_β and A_β chains, is identical in E^k_β and E^b_β. Thus, one could postulate that this 68–75 region of the Ia molecule contributes a major portion of the binding energy that defines the specificity of the T cell–receptor interaction with the E_β chain for those clones that show degeneracy on B10.A and B10.A(5R) APCs. If this hypothesis is correct, one would predict that the E^s_β molecule will show variability in this region of the molecule, since these T-cell clones are not degenerate on B10.S(9R); however, the E^s_β might possess identity with E^k_β in another hypervariable region of the molecule in order to explain the degeneracy on B10.S(9R) seen with 5–10% of the cytochrome *c* specific clones (Figure 1).

Although the identity of certain Ia-molecule hypervariable regions might help to explain violations of the MHC-restriction rule, it should be emphasized that in these cases it must be sequence variations in other areas of the Ia molecule that exert the Ia molecule's effect on the antigenic fine specificity of the T-cell response. This effect could occur in two ways (Figure 2). One is if the Ia molecule and the antigen physically contact each other during T-cell activation. This interaction could occur either prior to the approach of the receptor, if the Ia molecule by itself has a high enough affinity for the antigen, or only in the presence of the T-cell receptor, if the interaction is of low affinity. The alternative way in which the Ia molecule could influence antigenic fine specificity is through an allosteric effect on the receptor. This could come about if the conformation of a distinct antigen-binding site were influenced in different ways by different allelic forms of the Ia molecule when they interacted with a distinct Ia-binding site on the

receptor. Thus, this two-combining-site model would avoid any physical interaction between the antigen and the Ia molecule. Experiments designed to distinguish between these two models are described in the next section.

Before turning to these experiments, however, I want to describe an experiment by Hünig & Bevan (9) which suggests that antigen recognition by cytotoxic T cells is fundamentally similar to that of helper T cells. These investigators identified several cytotoxic T cell clones with an unusual dual specificity. The cells recognized one minor histocompatibility antigen in association with one class I molecule (H-2^k) and a second distinct minor histocompatibility antigen in association with a second allogeneic class I molecule (H-2^d). In the jargon of MHC-restriction; self plus X appeared the same as allo plus Y. The critical part of this experiment relative to receptor recognition was that neither of the reciprocal combinations, self plus Y or allo plus X, was recognized by the clone. Thus, in a finding similar to those of Kappler et al and Heber-Katz et al, the recognition of antigen was not independent of the recognition of the MHC-encoded molecule. If the same receptor on the cell recognized both combinations, then, similar to the

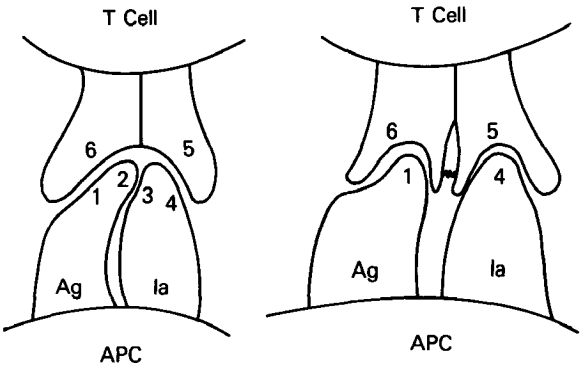

Figure 2 Two alternative models to explain the interaction between antigen and the Ia molecule during T-cell activation. The model on the left postulates a physical interaction between the antigen and the Ia molecule. This could occur before the T-cell receptor is introduced, if the interaction is of high affinity, or only after the receptor is introduced, if the interaction is of low affinity. In the latter case the T-cell receptor must have both chains (α and β) contribute to a single combining site as shown. The model on the right postulates an allosteric interaction between the antigen and the Ia molecule via the T-cell receptor. This is pictured as a "spring" connecting the two separate combining sites on the receptor. In this case no physical interaction occurs between the antigen and the Ia molecule. The numbers in both figures refer to the amino acid contact residues of the different components: 1 is the site on the antigen that contacts the T-cell receptor (epitope); 2 is the site on the antigen that contacts the Ia molecule (agretope); 3 is the site on the Ia molecule that contacts the antigen (desetope); 4 is the site on the Ia molecule that contacts the T-cell receptor (histotope); 5 is the site on the T-cell receptor that contacts the Ia molecule (restitope); and 6 is the site on the T-cell receptor that contacts the antigen (paratope).

conclusion reached for inducer T cells, this result implies that the specificity of antigen recognition by the receptor on cytotoxic T cells is influenced by the allelic form of the histocompatibility molecule being co-recognized.

Evidence for Functionally Distinct Subsites on the Antigen

A clear distinction between the two general models shown in Figure 2 to explain the nonindependent behavior of T-cell recognition is seen in the requirements imposed upon the antigen. In the physical interaction model, the antigenic determinant being recognized must have two distinct sites, one that contacts the T-cell receptor (epitope) and a second that contacts the histocompatibility molecule (agretope). In contrast, the allosteric model only requires one site, which contacts the receptor. Because the antigenic determinant in pigeon cytochrome *c* had been so well defined, my laboratory set out to find evidence for two distinguishable sites on the determinant.

Our initial observation in this area, like most scientific discoveries, was accidental. Dan Hansburg had derivatized pigeon cytochrome *c* with methyl acetimidate as a first step in coupling the 81–104 cyanogen bromide C-terminal fragment to a carrier protein (29). Acetimidization blocks the ε amino group of lysine, converting it to a methyl analog of homoarginine and preventing it from reacting with the coupling reagents (Table 1). However, problems in removing the blocking group without denaturing the carrier protein led us to try the acetimidyl derivative as an immunogen. It proved to be a powerful immunogen, although it was a poorly cross-reactive antigen when used to stimulate T cells primed to the native pigeon cytochrome *c* fragment 81–104 (100–1000-fold less potent). Surprisingly, when we attempted to map the antigenic determinant by stimulating with chemically derivatized cytochromes *c* from other species, we found that the pattern of cross-reactions observed was the same as that found when native cytochrome *c* was used as the immunogen. The fact that the patterns were similar suggested that the two sets of antigens (native vs acetimidyl derivatives) shared certain structural features. Yet at the same time the two sets of antigens were poorly cross-reactive with each other, suggesting that other structural features were different. This set of observations started us thinking about antigenic determinants having two distinct subsites.

To explore a hypothesis that there are two subsites, we turned to peptide synthesis in order to be able to create analogs that differed only in a single amino acid (30). Because acetimidyl derivatization modifies lysines, we initially focused on these residues. A short peptide containing the moth cytochrome *c* C-terminal sequence 88–103 was synthesized (Table 1) and found to have full stimulatory activity in both native and chemically

derivatized forms, although, as expected, each stimulated well only T-cell populations specific for their own form. This peptide contains two lysines located at residues 99 and 103 (Table 1). Because tuna cytochrome *c*, which has a serine instead of a lysine at position 103 (Table 1), did not stimulate after chemical derivatization, we postulated that the lysine at 99 must be the critical amino acid that was altered by acetimidization. To test this we made a synthetic analog with a glutamine at position 99 (Table 1). This peptide was very poor at stimulating T cells primed to native moth cytochrome *c* or to synthetic peptides having the normal lysine at position 99 (moth 86–90; 94–103 in Table 1). Shifts in the dose-response curves of 1000–10,000-fold were observed. However, when B10.A and B10.S(9R) mice were immunized with the Gln-99 analog, the T-cell populations elicited responded well to the Gln-99 analog and poorly to molecules containing a lysine at position 99. The reciprocal nature of the results with different T-cell populations in the presence of the same APCs, as well as a more recent observation that the same loss of activity of the Gln-99 analog is seen when paraformaldehyde fixed APCs are utilized (Z. Kovac, R. Schwartz, unpublished observations), both argue that it is not an alteration in antigen processing induced by substitutions at position 99 that accounts for the results. Thus, substitution of a glutamine (or an acetimidyl-lysine) for a lysine did not destroy the antigen but rather changed the specificity of the T-cell clones elicited by the immunogen. This property of changing the "memory" of the T-cell population is likely to result from stimulation of different clones of T cells bearing different antigen-specific receptors. If so, then position 99 is a good candidate for a site on the antigen (epitope) that physically interacts with these receptors.

Very different results were obtained when alterations were performed at position 103 (30). In attempting to understand the structural basis of the phenomenon of heteroclicity—i.e. the more potent nature of moth cytochrome *c* over pigeon cytochrome *c* when stimulating T cells primed with pigeon cytochrome *c*, we synthesized an analog of pigeon cytochrome *c* in which the alanine at position 103 was deleted (des-Ala-pigeon 81–104 in Table 1). This moved the C-terminal lysine from 104 to 103, making it identical to moth cytochrome *c* at this position. This single alteration converted the biological behavior of the pigeon fragment 81–104 to that of the moth fragment 81–103—i.e. the des-Ala-pigeon 81–104 was heteroclitic and just as potent as the moth fragment.

The phenomenon of heteroclicity is unusual in immunology and could have several different biological explanations. In the case of pigeon and moth cytochromes *c*, what is clear is that immunization with either molecule elicits the same family of T-cell clones, cells that respond to moth cytochrome *c* better than pigeon cytochrome *c*. In this sense, the immune

system of the B10.A mouse does not seem able to adapt to the difference between pigeon and moth cytochromes *c* the way it can easily alter its response to peptide analogs with substitutions at position 99. This suggests that position 103 might not be directly recognized by the antigen-specific T-cell receptor.

Evidence that position 103 of the antigen might instead contact the Ia molecule came from an examination of other immunological differences between pigeon and moth cytochromes *c* (24). First, the B10.A(5R) strain is a nonresponder to pigeon cytochrome *c* while moth cytochrome *c* elicits a T cell–proliferative response. In contrast, B10.A responds to both cytochromes *c*. Second, as mentioned earlier, T-cell clones and populations from either B10.A or B10.A(5R) can respond to pigeon cytochrome *c* in association with B10.A antigen-presenting cells but not B10.A(5R) antigen-presenting cells, whereas moth cytochrome *c* can be presented by either APC. These antigen-specific effects of the Ia molecule on the T-cell response were shown by Hansburg & Heber-Katz to involve only the area of the antigen around position 103 (31). They demonstrated that the des-Ala-pigeon 81–104 analog was immunogenic in B10.A(5R) and that it could be presented by B10.A(5R) APCs. Thus, deletion of the alanine at position 103 reverses effects caused by switching allelic forms of the Ia molecule. One possible explanation for these results is that the Ia molecule physically interacts with this part of the antigen during T-cell activation. We have tentatively proposed the term "agretope" for the region of the antigen that contacts the restriction element (which in this case is the Ia molecule, but for cytotoxic T cells would be a class I molecule) (24).

One of the puzzling outcomes of this subsite-specific mapping is how close in space the two parts of the determinant appear to be. Positions 99 and 103 are only 4 amino acids apart. Nonetheless, the biological data strongly argue that these subsites are functionally independent. When the lysine at 99 was changed to acetimidyl-lysine (AmLys), the T-cell clones elicited by the pigeon form of the chemical derivative still responded to the moth form in a heteroclitic fashion (29). Similarly, it was shown that clones specific for the Gln at position 99 manifested a heteroclitic response to the moth form of the molecule (31). In addition, for both Gln 99 and AmLys 99 the des-Ala-moth form and not the pigeon form, which has the alanine present, was strongly immunogenic in B10.A(5R). Thus, for three variables at position 99 (Lys, AmLys, and Gln), the biologic effects of the presence or absence of the alanine at 103 were the same.

Is cytochrome *c* an unusual case with no general application to the molecular aspects of T-cell activation? A review of the literature suggests not. A number of examples exist of experimental results that have as a possible interpretation a two subsite structure of the antigenic determinant

recognized by T cells (32–37). The oldest example is the classic Ir gene experiment of Levine, Ojeda & Benacerraf from 1963 (32). They observed that responder Hartley strain guinea pigs manifested delayed-type hypersensitivity (DTH) to a variety of haptens coupled to poly-L-lysine (PLL). In most cases the responses were specific for the immunizing hapten coupled to PLL—i.e. DTH could not be elicited by other haptens coupled to PLL. In modern terms we would say that noncross-reacting T-cell clones were being elicited by each hapten-PLL conjugate. The interesting observation made by Levine and Benacerraf was that nonresponder guinea pigs failed to respond to *all* of the hapten-PLL conjugates. This result suggested that these molecules shared some common structural element that could not be recognized by the nonresponder animals. The most likely element was the PLL backbone. Levine et al postulated that nonresponder guinea pigs could not process the PLL chain and thus did not generate an immunogen recognizable by the immune system. Today, however, knowing that Ir gene effects are caused by differences in allelic forms of histocompatibility molecules, one could just as easily interpret the data to suggest that PLL contains an agretope that favorably interacts with responder Ia molecules, but not nonresponder Ia molecules, to allow T-cell activation to occur. The situation is exactly analogous to that of pigeon cytochrome *c* and its interaction with the B10.A and B10.A(5R) Ia molecules. I predict that as more T-cell antigenic determinants are carefully dissected, the existence of two functionally distinct subsites will again be documented.

Structural Constraints on Subsite Mapping

One of the problems in identifying contact residues of the antigenic determinant, whether it be composed of one or two subsites, is the possible effect that substitutions at neighboring residues can have on the overall structure of the molecule. Such effects can indirectly alter the contact residues and thus lead to mistaken assignments. One might have thought that this would not be a problem with small peptides since they have always been thought to exist in so many conformational states that they are viewed as random coils. However, recent theoretical work on a number of short peptides suggests that despite the multiplicity of states attainable, the molecules do show tendencies for preferred conformations (38). In the case of cytochrome *c*, such an analysis has allowed us to understand a paradoxical result made in the T-cell activation system (39).

In attempting to determine the shortest moth cytochrome *c* peptide capable of stimulating a T-cell response (Table 1), we observed that a peptide composed of moth residues 94–103 could stimulate a proliferative response from T cells primed to pigeon cytochrome *c*, whereas a peptide composed only of moth residues 97–103 could not, even up to concen-

trations as high as 10 μM (30). This seemed surprising since the residues we had identified as important were the lysines at 99 and 103, both of which were contained in the 97–103 peptide. Our first thought was that residues between 94 and 97 were also important for contacting the T-cell receptor. However, substitutions at position 97 (D. Hansburg, T. Fairwell, E. Appella, R. Schwartz; and B. Fox, R. Schwartz, unpublished observations) and at position 95 (B. Singh, R. Schwartz, unpublished observations) did not have much affect on antigenic potency (10-fold) and in several experiments did not affect T-cell "memory." Therefore, an alternative explanation was considered by Pincus et al (35). An examination of the 94–103 sequence using energy minimization techniques and a computer program for calculating conformational energy potentials (ECEPP) showed that the preferred conformation of the pentapeptide comprising residues 94 to 98 was an alpha helix, whereas the preferred conformation of the C-terminal pentapeptide, 99–103, was not. In the combined structure, residues 99 to 103 shifted into an alpha helical conformation as part of the overall global minimum structure for the decapeptide. Thus, residues 94 to 98 appeared to be nucleating the formation of an alpha-helical secondary structure for the entire decapeptide. If the contact residues, 99 and/or 103, required an alpha-helical backbone conformation for correct orientation with regard to either the T-cell receptor or the Ia molecule, then it is easy to see how residues 94 to 98 could influence the antigenic potency of the peptide.

These theoretical calculations cannot make absolute predictions about the number of molecules in a given conformation as they do not take into account solvent effects or entropy considerations. Nonetheless, they are of value in pointing up relative conformational preferences and at what transition point they are likely to occur. In this regard, the theoretical calculations demonstrated that by a chain length of 7 (residues 97–103) the peptide was already showing an alpha-helical preference by approximately 2 kcal (M. Pincus, unpublished observations). Based on this, we predicted that 97–103 should stimulate T cells if used at high enough concentrations. This prediction has recently been confirmed with the sensitive normal T-cell clone, AE7, which was stimulated by the 97–103 peptide at a 100 μM concentration (B. Singh, R. Schwartz, unpublished observations). Thus, the amino acids at positions 94, 95, and 96 are not essential contact residues, but instead appear to affect specificity by contributing to the overall conformation of the molecule.

Given these structural effects, how then can one map out epitopes and agretopes on any given antigenic determinant. This is a difficult task that clearly cannot be achieved by making only a few amino acid substitutions. However, a strategy can be followed that should give the general location of

the subsites. First, we have found that even conservative substitutions in the epitope (acetimidyl-Lys for Lys) have dramatic effects on potency. This is presumably because the T-cell receptor has been selected to recognize this region and therefore the maximum bonding energy requires exactly the right fit. Having located a potential site, one must next test to see if the T-cell population can adapt to substitutions at this site. This is done by immunizing with the substituted analog and determining whether the elicited T-cell population is specific for the immunogen. Although such a test can usually pinpoint an epitope, a number of cautions should be noted. One is that the immunizing analog must be absolutely pure. If contaminants are present, for example from incomplete or side reactions in solid-phase peptide synthesis, then these may prove to be immunogenic and elicit totally unrelated T-cell clones, giving a false positive result. This problem can be avoided by rigorously purifying the peptides by high-pressure liquid chromatography. A second problem that is more difficult to control is the flexibility of the T-cell repertoire. At the present time we do not know how expansive or adaptable this repertoire is. If it is too large, then even a small peptide might contain several recognizable determinants. This will make mapping difficult, because effects of substitutions on one determinant might be masked by responses to the other determinants. On the other hand, if the repertoire is small and not very flexible, then some substitutions may not be adapted to, giving false negative results. So far we have found the T-cell repertoire to be quite flexible and, therefore, we usually take precautions to minimize the number of antigenic determinants. This is done by choosing antigens that are poorly immunogenic in most inbred mouse strains but that elicit a good response in one or two strains. Then, by synthetic chemistry, we reduce the peptide to the smallest possible size capable of stimulating a full response. Working with such antigens we find that the T-cell responses are usually simple enough so that any complexities that do arise can be easily observed and understood. For example, the Gln substitution at position 99 elicited two types of T-cell clones on immunization of the B10.A mouse (30). One was of apparent high affinity and cross-reacted with analogs of the native moth cytochrome *c* containing a lysine at 99. The other set of clones appeared to be specific for the Gln at 99 as they did not show cross-reactions with the native moth cytochrome *c* analogs. Only one of these clones was found in the B10.S(9R). This strain made a response that was entirely specific for the Gln 99 substitution. Thus, in this situation we could clearly conclude that position 99 was behaving with the characteristics of an epitope. The clone(s) in the B10.A that responded to Gln and Lys equally well has not been further characterized. However, we would postulate that it probably represents a variant whose receptor contacts a different residue on the peptide.

Identification of the agretope requires a completely different strategy. Substitutions in this area of the determinant also produce a decrease (or an increase) in antigenic potency although usually the decrease is not as dramatic as for substitutions at the epitope. In addition, substitutions at the agretope are not "remembered"—i.e. immunization with the substituted analog elicits the same T-cell clones as the native peptide. Our usual clue that a residue is part of the agretope has come from observing the phenomenon of heteroclicity—i.e. when a substitution increases the potency of the molecule. However, it is important to note that other biologic events might give rise to this effect. For example, substitutions at a distance from the antigenic determinant could affect antigen processing leading to the accumulation of greater concentrations of the fragments required for stimulation. To avoid such problems one must demonstrate that the heteroclitic effect can be obtained with the small synthetic peptide possessing the single amino acid substitution, preferably when aldehyde-fixed APCs are used to prevent processing.

Having located a potential residue in the agretope by this procedure, one must next demonstrate that the substitution is affecting the interaction with the Ia molecule and not simply disturbing the overall structure of the determinant. To accomplish this, one must show a relationship between changes in the histocompatibility molecule and changes at the putative agretope. This can be done by studying Ir-gene effects (responsiveness and nonresponsiveness in a set of MHC-congenic mouse strains) and effects on the T cells's fine specificity for antigen using degenerate T-cell clones. The latter must be searched for by screening T-cell clones for responsiveness to the heteroclitic antigen in association with as many antigen-presenting cells of different MHC haplotypes as possible. Having found such a system, one assesses the ability to present both antigens with both presenting cells to look for changes in antigenic potency with changes in the allelic form of the Ia molecule. Finally, new substitutions must be introduced at this site in the peptide and assessed for their ability to stimulate with the two Ia molecules. Under these conditions, a pattern in the effects of the peptide substitutions can usually be discerned, although sometimes such patterns are only seen with the less potent of the two Ia molecules.

At this point one has only established a correlation between amino acid substitutions at particular places in the antigen and certain types of biological effects on an immune response. Unfortunately, one can only infer from an indirect biological assay what are the biochemical events that occur during activation. To circumvent this problem one needs to do direct measurements on the receptor-antigen-Ia molecule complex in order to discern the physical relationships among the various components. At the present time this appears to be a formidable task. However, recent advances

in cloning the genes for the T-cell receptor and the histocompatibility molecules suggest that it may eventually be possible to set up an in vitro system to investigate such questions.

Antigen Competition Studies

If the Ia molecule and the antigen physically interact, and if a specific subsite (agretope) exists on the antigen that is capable of mediating this interaction, then one might predict the existence of analogs of the antigen with amino acid differences at the epitope that make them poorly cross-reactive for T-cell stimulation but still allow them to compete for binding to the site on the Ia molecule (desetope) that interacts with the agretope. The first report of such a phenomenon was by Werdelin (10), who showed that the T cell–proliferative response to DNP-PLL could be blocked by the synthetic copolymer poly-$(Glu^{20}Lys^{80})_n$ [GL]. Strain-2 guinea pigs respond to both DNP-PLL and GL, but the T cells specific for DNP-PLL are not cross-stimulated by GL. Addition of GL at the initial antigen-pulsing step, when the antigen-presenting cell was first exposed to DNP-PLL, was sufficient to block subsequent stimulation of the T cells specific for DNP-PLL. This result suggested that competition was occurring at the level of the antigen-Ia molecule interaction. Of note was the fact that one could first pulse with GL, wash, and two hours later still block T-cell stimulation with DNP-PLL. This suggested that the antigen–Ia molecule interaction was of relatively high affinity.

One of the problems with such studies is that the competitor may be toxic at the high concentrations used for blocking. Although GL did not block OVA primed T cells, and other highly positively charged molecules such as poly-L-arginine did not block the DNP-PLL primed T cells, the possibility of a synergistic toxic effect between GL and DNP-PLL on the strain-2 APC is hard to rule out. In addition, the blocking was not reciprocal—i.e. DNP-PLL did not significantly inhibit the GL response. However, these objections have largely been overcome by the studies of Rock & Benacerraf (11). They discovered that some T-cell hybridomas specific for poly-$(Glu^{60}Ala^{30}Tyr^{10})_n$ [GAT] in association with Ia molecules of the *d* haplotype could be inhibited from releasing IL-2 by exposing the antigen-presenting cells to poly-$(Glu^{50}Tyr^{50})_n$ [GT] at the time of antigen-pulsing with GAT. Because other T-cell hybridomas specific for GAT in association with Ia molecules of the *b* haplotype were not inhibitable by GT, a control experiment could be carried out using F_1 antigen-presenting cells. Such APCs, pulsed with GAT and GT simultaneously, were capable of stimulating the Ia^b-restricted hybridomas but not the Ia^d-restricted hybridomas. This selective blocking strongly argues against not only toxicity to the APC but also all nonspecific mechanisms of blocking such as

inhibition of GAT uptake by the cells (unless the Ia^b-restricted T-cell hybridoma bears antigen receptors that have a much higher affinity for the Ia molecule and antigen, making it less susceptible to decreases in APC function).

The best evidence that the blocking actually occurs at the level of the antigen-presenting cell also comes from the experiments of Rock & Benacerraf (11). The alternative possibility that had to be eliminated was that the competitor and the Ia molecule interacted with the T-cell receptor in a nonproductive manner and thus competed by binding to receptor molecules rather than by binding to Ia molecules. This possibility was addressed in two ways. One was by pulsing separate populations of APCs with GAT and GT and then mixing them together in the stimulation assay. The failure to see inhibition suggested that GT could not block T-cell receptors when on presenting cells other than those bearing GAT. However, the most elegant and convincing experiment was the blocking observed with a dual-reactive clone. One T-cell hybridoma displayed autoreactivity to syngeneic Ia molecules alone as well as antigen-reactivity to GAT in association with the $A^d_\beta : A^d_\alpha$ Ia molecule. Using the same antigen-presenting cell, the antigen-specific reactivity could be blocked by GT, but the autoreactivity was unaffected. If the same receptor (or part of a receptor) is used for both specificities, then this experiment strongly argues against competition at the level of the T-cell receptor.

Since these studies were published, several other laboratories have tried to reproduce these findings in their own antigen systems. Although one group has recently reported success in the ABA-tyrosine system (40), several groups have failed. The most surprising result perhaps is in the cytochrome *c* system. Here there is functional evidence for Ia molecule–antigen interaction and a fairly clear picture of the epitope and agretope. Yet the Gln [Q] 99 analog of moth cytochrome *c* 93–103 (Table 1), which is poorly if at all cross-stimulatory for T-cell clones specific for the native molecule containing Lys 99, did not block stimulation of these clones by molecules containing Lys 99 even at a 3000-fold molar excess (B. Fox, R. Schwartz, unpublished observations). In contrast, the synthetic polypeptide blockers (GL, GT) competed at microgram amounts roughly similar to the doses used for the stimulators.

I do not feel that the negative results at all invalidate the positive results showing blocking. However, a discussion of the discrepancies is clearly warranted. One unknown variable is the relative affinity of the interactions between the Ia molecule and the antigen in the different systems. Judging from the concentrations required for inhibition in the GAT and DNP-PLL systems, as well as the ability to compete prior to introduction of the T cells, one might suggest that the binding constant is relatively high and that

antigen–Ia molecule complexes can form in the absence of the T-cell receptor. In contrast, the Ia molecule–antigen interaction in the cytochrome *c* system might be of very low affinity and only be detectable in the presence of the T-cell receptor. Therefore, in order to compete with the native molecule, one might require much higher concentrations than the 3000-fold molar excess tested to see competition. Alternatively, all the relevant antigen–Ia molecule interactions might be of comparably low affinity, but the charged polymers used in the GT and GL studies might have additional cell-surface binding sites that are of high affinity. These could bring the competitors into close proximity with the Ia molecules and keep them there at effectively high concentrations. The blocking would then occur as a second step involving a low-affinity, but still selective, interaction. No matter which interpretation (or others) one favors, I think the competition experiments are consistent with the other observations mentioned in this review, all strongly suggesting that the Ia molecule and the antigen physically interact with one another at some point during the T-cell activation process.

MODELS FOR THE INTERACTION OF THE T-CELL RECEPTOR, THE Ia MOLECULE, AND ANTIGEN

The experiments of the last few years that I have summarized place fairly strong constraints on any model of the T-cell receptor for antigen. Structurally the molecule appears to be a disulfide-linked heterodimer with each transmembrane chain having two extracellular domains analogous to one constant region and one variable region of immunoglobulin. Because of the strong sequence homology to immunoglobulin, it seems reasonable to assume that the three-dimensional structure will be similar. If so, there are only two general ways one can orient the chains. Either the two variable regions will come together to form a single combining-site pocket similar to an immunoglobulin heavy and light chain interaction, or the two variable regions will be separate from one another giving the potential for two distinct combining sites similar to the orientation of the variable regions of two heavy chains (Figure 2). The presence of an exon with homology to the hinge region of immunoglobulin tilts the structural data slightly in favor of the analogy to two immunoglobulin heavy chains, where this region facilitates the separation of the two chains.

On the other hand, all the functional data strongly argues in favor of a single combining site model. Scrambling of the two chains of two receptors with different specificities seldom if ever produces functional hybrid molecules. Furthermore, data from the pigeon cytochrome *c*–proliferative system (8, 25) strongly suggests that the histocompatibility molecule and

the antigen physically interact. This conclusion is supported by the data of Hünig & Bevan (9) and by the observations of antigenic competition at the level of the antigen-presenting cell (10, 11). The "interaction" results could be explained in a two-combining-site model by postulating an allosteric mechanism of interaction. However, such a model would not predict the existence of functionally distinct sites on the antigen (agretope and epitope) and cannot simply explain the antigen-competition experiments. Therefore, at the present time, only the single-combining-site models appear to explain all the data.

The most straightforward single-combining-site model is one in which the two chains of the T-cell receptor interact with one another to form a structure similar to that formed by the interaction of a heavy and a light chain of immunoglobulin (Figure 3A). The distal end of such a receptor

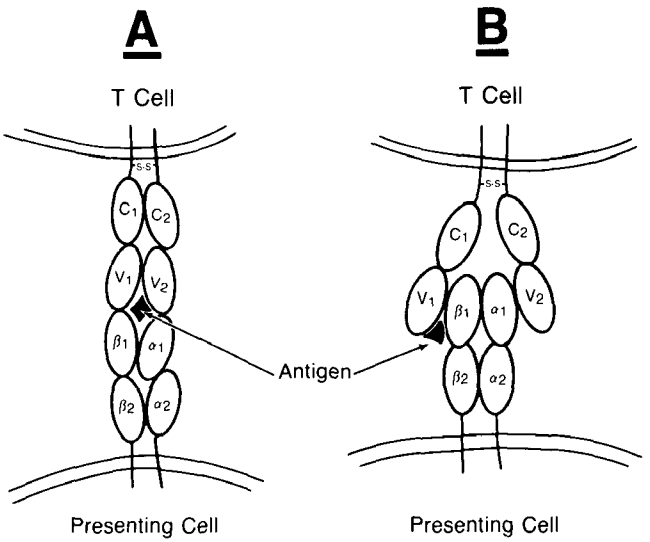

Figure 3 Two single-combining-site models for the interaction of the T-cell receptor, the Ia molecule, and antigen. Model A depicts the two chains of the T-cell receptor coming together to form a single combining site in a way analogous to that of the heavy and light chains of immunoglobulin. The receptor combines with both chains of the Ia molecule to form the antigen-binding pocket. The affinity of interaction between the Ia molecule and the antigen will determine whether Ia-antigen complexes can form in the absence of the receptor. Model B depicts only one chain of the receptor interacting with only one chain of the Ia molecule to form an antigen-binding pocket. In this version of the Norcross & Kanehisa model (7), the second chain of the receptor interacts with the other chain of the same Ia molecule. See reference 7 for an alternative form of Model B that leads to Ia molecule cross-linking. Note that model B clearly predicts the possibility that a single T-cell clone could have specificity for two different antigens, unless, as recently suggested (6), the alpha chain of the T-cell receptor is not pleiomorphic.

would interact with the distal ends of both chains of the class II molecule and the antigen would bind to the combining pocket formed by this macromolecular interaction. In this way antigen could contact the Ia molecule as well as the T-cell receptor. Thus, both molecules could contribute to the specificity of the binding.

An alternative model recently proposed by Norcross & Kanehisa (7) starts from the premise that the histocompatibility molecules also have a domain structure similar to that of immunoglobulin. The new twist in their hypothesis is the postulate that the T-cell receptor's recognition of the Ia molecule involves a domain-domain interaction similar to that of a heavy- and light-chain variable-region interaction. Thus, the external domains (α_1, β_1) of the Ia molecule in any individual are simply structurally invariant light chains. The T-cell receptor provides the structurally variant heavy chains. Antigen binds to the combining pocket just as it would in immunoglobulin and, therefore, both molecules can influence specificity (Figure 3B).

At this point the data do not compellingly support one model over the other, and I think either is a likely possibility. In fact, the two are difficult to distinguish experimentally except in the extreme forms shown in Figure 3. If antigen always fully occupies the combining pocket, then model A predicts that the α and β chains of both the Ia molecule and the T-cell receptor should all influence the specificity of T-cell activation by a single antigen. In contrast, model B predicts that any given antigen will only be recognized by a single V region and a single chain of the Ia molecule, and each receptor should be capable of recognizing two distinct foreign antigens. Correlations between the specificity of activation and sequence variability in the T-cell receptor and the Ia molecule (for degenerate clones) could provide the data required to make the distinction between these two models. However, it is highly likely in model A that some antigens will not contact all four components when bound. If the frequency of such cases is high, it might be impossible to distinguish model A from model B. Furthermore, it would be difficult to tell whether a cell with two different antigen specificities possessed a model-B receptor rather than a model-A receptor with a degeneracy in its combining site. One would need to have monoclonal anti-Ia or anti-receptor antibodies that could selectively inhibit particular chains from functioning or be able to separate the receptor and Ia chain pairs and show that each is responsible for activation by one of the two antigens. Finally, if the α chain of the T-cell receptor is not pleiomorphic, as has recently been suggested (6), then even in model B it is likely that there would be only a single antigen combining site. Thus, it seems reasonable to state that only a structural analysis of the isolated receptor–Ia molecule–antigen complex will definitively distinguish between these models.

EVOLUTIONARY ORIGINS OF MHC-RESTRICTED T-CELL RECOGNITION

The concept that the immune system evolved from a primitive cell-cell interaction mechanism is an old idea in immunology (41, 42). In molecular terms one can imagine an early like-like protein interaction, based on a head-to-tail interaction, evolving into a receptor-acceptor pair as the basis of cell-cell recognition. This could in turn, through duplication, genetic drift, and selection, evolve into a set of receptor molecules that have acquired the ability to distinguish "foreign" from "self," first presumably at the level of the species (allogeneic recognition) and then at the level of the individual (antigen recognition).

In this scheme one of the things not totally clear is why the immune system would preserve the vestiges of the cell-cell interaction mechanism in its new role of recognition of foreign molecules. In other words, why maintain MHC-restricted recognition of antigen? An early teleologic argument presented by Langman (42) as well as others was the need to identify intracellular parasites by the foreign products they put out on the cell surface. If one recognized just the free antigen, then the immune system would be distracted by soluble circulating forms of the molecule and not be able to identify and destroy the source of the problem. This argument has much substance. However, my feeling is that it is only part of the story.

I think a more critical element centers around the problem of self-nonself discrimination. Each individual must go through a process of ontological selection in which it learns what molecular shapes constitute self; it must then set a biological response threshold to prevent reaction against such molecules. The system must be finely tuned to avoid self-reactivity and yet at the same time be allowed to pick up the subtle variations in shape that constitute the foreign molecules. Such discrimination is normally the property of high-affinity receptors, which contact even small molecules at multiple sites and can thus discriminate among a variety of different analogs. The evolution of such a receptor to recognize a peptide hormone or a neurotransmitter is relatively simple; however the evolution of a family of such receptors, when the shapes to be recognized are only decided upon at the level of the individual organism, seems a much more formidable process. One cannot solve this problem with an extensive random mutation mechanism because the possibility of producing receptors with anti-self specificity is too great.

I propose that nature's solution to this problem lies in MHC-restricted recognition. If recognition occurs by either of the single-combining-site models discussed in the previous section, then the spatial constraints of antigen binding in the presence of an invariant Ia molecule are such that

even a subtle difference in a T cell–receptor contact residue (e.g. Lys 99) can be detected. In a system that can recognize small fragments of antigen (in the extreme case, ABA-Tyr) there often is only one contact residue to be discerned by the T-cell receptor, thus simplifying the problem of the V-region diversity required for sufficient specificity. Focusing antigen recognition around the recognition of a self-molecule allows affinity thresholds to be set using a small number of V genes. Perhaps in early evolution the V segment was invariant and only provided sufficient energy to bind to the MHC molecule. Addition of the D and J elements may have constituted the first evolutionary level of variability for antigen recognition. Subsequent duplication of the V genes may then have allowed these genes to diversify, enabling them to contribute also to antigen specificity. Finally, perhaps most importantly, the fact that the histocompatibility molecule is a cell-surface protein on the antigen-presenting cell confers the great thermodynamic advantage of multimeric presentation of antigen. This could allow small differences in binding energy between foreign and self molecules to be amplified into an all-or-nothing transition for T-cell activation. Some of these ideas should be testable in the near future as in vitro systems become available for directly measuring interactions among the T-cell receptor, antigen, and the histocompatibility molecule.

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CONTENTS

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INTERLEUKIN 1: AN IMMUNOLOGICAL PERSPECTIVE[1]

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INTRODUCTION

Interleukin 1 (IL-1) is a hormone-like polypeptide that apparently performs many roles in inflammation and immunity. Originally described as a product released from activated macrophages (MΦ), IL-1–like factors have now been detected in the culture supernatants of many types of cells.

Historically, IL-1 has been rediscovered a number of times based on the diversity of its actions: the endogenous mediator of fever (1), the comitogen for thymocytes (2), the stimulant of the acute-phase response (3), cartilage resorption (4), and muscle wasting (5). Each of these properties was initially ascribed to a distinct soluble factor, usually with its own acronym. Only recently have they all been attributed to a single mediator, IL-1.

The immunological discovery of IL-1 sprang from serendipitous findings during the course of early studies on suppressor T cells (Ts). The possibility was being tested that Ts, which had been elicited in mice by sheep erythrocyte (srbc) antigens, might suppress the mitogenic response of T cells stimulated by phytohemagglutinin (PHA). One experimental control consisted of adding human erythrocytes to mouse thymocytes plus PHA. These erythrocyte preparations were often (but not always) quite mitogenic for thymocytes. This variability in stimulation of mitogenesis was eventu-

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ally attributed to a loss of stimulatory capacity with aging in vitro of the human blood preparations. The labile mitogenic component was not the erythrocyte, but the blood monocyte (I. Gery, R. K. Gershon, personal communication). This finding eventually led to the description of a soluble mediator (produced by monocytes) that augmented murine thymocyte mitogenesis (2, 6), and this property remains the operational definition of IL-1 today.

In recent years, a great diversity of cellular sources and actions of IL-1 has been appreciated, removing it from the exclusive domain of immunologists, as well as prohibiting the possibility of a comprehensive review here [for an extensive review, see (7)]. While reminding the reader of the broader physiological setting into which IL-1 fits, we will restrict our detailed coverage to areas of direct interest to immunologists.

THE IL-1 MOLECULE

IL-1 is a polypeptide as determined by its sensitivity to a variety of proteases (8) and by its loss of activity following covalent modification of arginine residues (9). Although the possibility that IL-1 is a glycoprotein has not been excluded, there is currently no direct evidence that carbohydrate residues are present or essential to IL-1 bioactivity (10, 11). The molecular weight of the extracellular or secreted form of IL-1 is approximately 15 K in all species examined thus far (10, 12, 13) (Table 1). However, recent experiments have shown that the mRNA transcript for IL-

Table 1 Properties of murine, human and rabbit IL-1

	Murine	Human	Rabbit
Size (M_r)	14,000	15,000	14,400 11,600
Isoelectric points	4.9, 5.0, 5.1	5.2, 5.4, 6.0, 6.8	4.6, 7.4
Bioactivity			
a. murine thymocyte proliferation activity	+	+	+
b. endogenous pyrogen activity	+	+	+
c. synovial cell activation	+	+	+
d. fibroblast proliferation activity	+	+	N/A[a]
e. IL-2 induction	+	+	N/A
f. SAA induction	+	+	N/A
Cross-reactivity with goat anti-mouse IL-1	+	±	N/A
Chymotrypsin sensitivity	−	+	N/A

[a] N/A: not available.

1 codes for a molecule at least twice this size (35 kilodaltons) that is subsequently processed intracellularly into the smaller, extracellular form (14). Intracellular and extracellular species having considerably larger molecular weights (60–250 K) have been reported sporadically (13, 15, 16) but probably represent either aggregates with contaminating proteins or, possibly, aggregates of IL-1 monomer resulting from mixed disulfide bridges between free cysteine residues. A high-molecular-weight carrier protein for IL-1, such as those described for other circulating hormones, might also explain these high-molecular-weight forms, but no direct evidence for such a carrier yet exists. Interestingly, a recent study of IL-1–like molecules in urine suggests that IL-1 may be processed further to peptides of only two and four kilodaltons without complete loss of bioactivity (17). The resistance of IL-1 bioactivity to certain proteases also raises this possibility (8, 10). Determination of the precise relationship between these small urinary IL-1–like molecules and the IL-1 found in culture supernatants awaits their purification and biochemical characterization.

The purification of IL-1 has met with difficulty, partly because, unlike other circulating hormones such as insulin or epidermal growth factor, it is not stored in glands or tissues. Thus it has been necessary to purify IL-1 from the supernatants of cultured cells (see below) where, even with the use of superinduction techniques, it is only found in low picomolar amounts. Another difficulty, particularly in the case of human IL-1, has been the identification of cell lines capable of secreting IL-1 under serum-free conditions. A cloned macrophage line, P388D1, has proven to be a reliable source of murine IL-1 since its description as such in 1977 (18). A recent report suggests that a clone of THP-1, a human histiocytic lymphoma line, may finally provide a human equivalent to P388D1; it appears to secrete appreciable levels of IL-1 under serum-free conditions when stimulated with lipopolysaccharide (LPS) or silica (19). Unlike the IL-1 produced by the parent line, the IL-1 produced by the THP-1 clone consists of a single size/charge species, a feature that should aid considerably in its purification.

Murine IL-1 was initially purified from the supernatants of phorbol myristate acetate (PMA)–stimulated P388D1 cells using a protocol consisting of ammonium sulfate fractionation, phenyl Sepharose hydrophobic chromatography, gel filtration chromatography, and preparative isoelectro-focusing (IEF) (20). The isolated material exhibited microcharge heterogeneity with isoelectric points (pI) of 4.9, 5.0, and 5.1 (Table 1). The partial amino acid composition of the pI 5.0 species was determined but has not been confirmed. More recently, P388D1-derived IL-1 has been isolated by immunoaffinity procedures using a heterologous antiserum prepared in goats against murine IL-1 (21). When the isolated material was analyzed by simple electrophoresis, at least seven discrete species were observed. When

analyzed in a second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), these species were found to have different molecular weights in the range of 13–17 K. This finding plus the previously mentioned observation that IL-1 is translated as a 35-kilodalton polypeptide, has suggested that the observed charge heterogeneity is not due to different gene products but to various degrees of proteolytic cleavage of a single parent molecule. The different charged species of rabbit IL-1 likewise have different molecular weights (13).

In contrast to the material derived from P388D1 cells and mouse peritoneal macrophages, IL-1 derived from human peripheral blood monocytes exhibits macrocharge heterogeneity with major pIs of 5.2, 5.4, 6.0, and 6.8 (8) (Table 1). The molecular basis of this charge heterogeneity remains unclear. Posttranslational modifications of a peptide with carbohydrate residues usually result in a less discrete distribution of charge species and thus are not likely to be responsible. Whatever the explanation for the charge heterogeneity, all of the species exhibit the same broad spectrum of bioactivities (see below) and therefore probably have certain critical domains in common. An additional possibility is that these species are the products of a gene family and not simply the result of variable proteolytic cleavage reactions; this interpretation will be put to the test by the isolation of cDNAs coding for human IL-1 and the purification of the more acidic species of human IL-1, studies currently in progress in a number of laboratories.

Human IL-1, derived from peripheral blood monocytes, has been partially purified using a protocol employing ultrafiltration, IEF, and preparative SDS-PAGE (22), or by immunoaffinity chromatography using a polyspecific rabbit antiserum containing antibodies to human IL-1 (23). More recently, the pI 6.8 species of human IL-1 has been purified to homogeneity by high-performance anion and size-exclusion chromatography (24) (Table 2). The isolated material was pure as determined by SDS-PAGE, analytical IEF, and reverse-phase high-performance liquid chromatography (HPLC). Using absorption at 210 nm as a measure of IL-1 protein concentration, the purified material was found to give half-maximal stimulation of thymocyte proliferation at a concentration of 1×10^{-10} M. Using amino acid composition as a more accurate measure of IL-1 protein content, we have recently revised this figure to 2×10^{-11} M. Similarly, the specific activity of the purified material has been revised from 7.6×10^6 half-maximal units per mg of IL-1 protein to 1.5×10^7 units per mg. Interestingly, the amino acid composition of the pI 6.8 species of human IL-1 differs considerably from that reported for the pI 5.0 species of P388D1 murine IL-1 (J. Schmidt, manuscript in preparation).

Table 1 compares some of the biological, immunological, and bio-

Table 2 Purification of normal human IL-1[a]

Step	Total activity[b] (units $\times 10^{-3}$)	Total protein[c] (mgs)	Specific activity (units/mg)	Times purified	Percent yield
1. Crude concentrated culture supernatant	406	147	2.7×10^3	—	100
2. Preparative HPLC anion exchange chromatography	146	0.52	2.8×10^5	102	36
3. Analytical HPLC anion exchange chromatography	138	0.021	6.5×10^6	2380	34
4. HPLC size exclusion	122	0.016	7.6×10^6	2761	30

[a] The results are those of a representative run in which 5000 ml of crude culture supernatant were processed.
[b] Activity was measured in the thymocyte proliferation assay.
[c] Protein was measured by calibrating the peak integrator with known amounts of bovine serum albumin.

chemical properties of murine, rabbit, and human IL-1; all three demonstrate the full spectrum of IL-1 bioactivities in so far as they have been tested. Whether all of these functions can be mapped to a single domain on the IL-1 molecule remains to be determined. Interestingly, certain of the small urinary IL-1–like molecules mentioned above demonstrate thymocyte proliferation activity but not fibroblast proliferation activity (17). These findings raise the possibility that different structural domains may subserve separate functions. Only the development of receptor binding assays can enable us to determine whether murine and human IL-1, and their various charged species, have the same affinity for putative binding sites on target cells from different species. Nevertheless, the observations that both murine and human IL-1 stimulate half-maximal murine thymocyte proliferation at a concentration of approximately 10^{-11} M suggests that both of these IL-1s bind to the murine IL-1 receptor with similar affinity. Murine and human IL-1 do, however, differ serologically, in that a highly specific antiserum to P388D1-derived murine IL-1 blocked the activity of murine IL-1 in the thymocyte assay much more efficiently than it blocked human IL-1 activity (21). Other less well-characterized heterologous antisera to rabbit (25) or human IL-1 (23) have not been extensively tested for their ability to block murine IL-1 activity. Another important biochemical difference between murine and human IL-1 is their differential susceptibility to inactivation by chymotrypsin: Whereas murine IL-1 is chymotrypsin-resistent, human IL-1 is inactivated (8, 10, 26). This finding is not surprising in light of the amino acid compositional data alluded to above.

PRODUCTION OF IL-1

In the laboratory, the macrophage/monocyte has served as the standard IL-1 producer. This lineage of cells, while no longer the sole source of IL-1, remains the most prolific producer, and does so in response to a remarkably wide variety of stimuli [reviewed in (7)] as shown in Table 3. We must marvel at the ability of the MΦ to respond to so many diverse chemical structures, particularly the microbial products, whose number underscores the probable importance of MΦ-derived IL-1 in host defense. One of the most remarkable of these activating agents, from the standpoint of potency, is the lipid A molecule, the active component of LPS, a constituent of the cell wall in gram-negative bacteria. As little as 25 pg/ml of lipopolysaccharide triggers IL-1 release from rabbit monocytes (46).

While little is known of the mechanisms employed by the MΦ in reacting to these diverse substances, surface receptors may generally be involved. For example, stimulation by particles must occur at the MΦ membrane,

Table 3 Stimuli of IL-1 release from monocytes

Immunological	
activated T cells	—cell contact, Ia-restricted
	—lymphokine (colony-stimulating factor)
immune complexes	
C5a	
Microbial	
gram-negative bacteria	—endotoxin (lipid A)
gram-positive bacteria	—cell walls, muramyl dipeptide, exotoxins
yeast	—cell walls (zymozan)
virus	—hemagglutinins, double-stranded RNA
Other	
silica crystals	
urate crystals	
phorbol myristate acetate	

since blocking the phagocytic uptake of spirochetes (47) or of urate or silica crystals (48) does not inhibit their stimulatory capacity. $C5_a$ recognition presumably occurs via receptors that are known to occur on the MΦ membrane. The Ia membrane glycoproteins may participate in recognizing stimuli delivered by T cells in the presence of antigens or lectins as well as the stimulation by LPS in the absence of T cells (49, 50).

Following the membrane event, an obligatory rise in intracellular calcium occurs (51), and augmenting intracellular calcium using the ionophore A23187 has been observed to enhance IL-1 production (52). This may be accompanied, as with other membrane receptor systems, by activation of c-kinase, since PMA, whose target is c-kinase, induces IL-1 release (53). Breakdown of arachidonate from the cell membrane may also occur during the first hour following stimulation, since inhibiting the lipoxygenase arm of arachidonate metabolism during this early period (but not later) blocks IL-1 release (54).

Most evidence indicates that IL-1 is not preformed; hence, de novo syntheses of RNA and protein are required. Specific mRNA for IL-1 appears by 2 hr after stimulation (detected by injection of frog oocytes), and secreted IL-1 protein is seen by three hr (55). However, prior to secretion, some findings indicate the occurrence of a large precursor peptide (displaying the full complement of IL-1 biological activities), which may then be cleaved prior to its extracellular appearance (14, 16). There are also indications of separate controls over the phases of IL-1 synthesis and IL-1 release, since some stimuli are particularly effective at causing intracellular accumulations of IL-1, while others are more potent at inducing release from the cell (56, 57).

One of the big surprises in IL-1 research came when, after a decade of studying monocyte IL-1, it was discovered that other types of cells are also capable of producing IL-1–like factors comitogenic for thymocytes. The list of such nonmonocytic IL-1 producers, as reviewed previously (58), is steadily growing. It now includes keratinocytes, kidney mesangial cells, corneal epithelium, stimulated B lymphocytes, large granular lymphocytes, fibroblasts, astrocytes, glioma cells, and endothelial cells. A number of transformed cell types also produce IL-1, including monocytic leukemias from mouse (P388D1 and J774) and man (THP-1, U937), human melanoma, E-B virus–transformed human B lymphocytes, a dendritic cell line, gliomas, and transformed mouse keratinocytes (PAM 212). Although some of the IL-1–like activities derived from non-MΦ have been partially purified, their relationship to monocyte IL-1 will be clear only when they are sequenced.

The stimuli that release IL-1 from normal non-MΦ IL-1 producers are less well characterized than those for MΦ. In the case of keratinocytes and kidney mesangial cells, for example, growth of cells in vitro is accompanied by IL-1 release, with no apparent requirement for other stimuli, although LPS increases the production from keratinocytes and is also a stimulant of IL-1 production from B lymphocytes (see below).

The biological significance of so many cells being capable of IL-1 production may relate to supporting immune or inflammatory reactions in the tissues populated by these cells. On the other hand, IL-1 may, in addition to its immune/inflammatory roles, serve as a common means of communication between cells of diverse types since, as discussed in the next section, many different cell types can respond to IL-1.

ACTIONS OF IL-1

General Actions: From Inflammator to Soporific

Recent findings from many laboratories have converged on IL-1, implicating it as a principal mediator of inflammatory responses. Thus a broad spectrum of responses of host tissues that are hallmarks of acute and chronic reactions to microbial products and to immune challenge have now been attributed to the IL-1 molecule. Some of these responses are summarized in Table 4, illustrating the broad span of reactive targets, including cells of such diverse origins as lymphocytes, fibroblasts, and hepatocytes. A wide range of effective distances between the IL-1 producer and the IL-1 target are also observed. IL-1 can act as a local mediator at the site of inflammation, as in synovia; it can also act as a circulating hormone, as in its action on the hypothalamus (inducing fever) or on hepatocytes (eliciting the production of acute-phase reactants). Furthermore, behavior

Table 4 Actions attributed to monocyte IL-1

	Target	Action	References
Growth and differentiation	T cells	lymphokine release	27, 28
	B cells	cofactor in proliferation/differentiation	29, 30, 31
	fibroblasts	proliferation	12
	natural killer cells	augments cytotoxicity	32
Inflammation	neutrophils	chemotaxis, degranulation, bone-marrow release	33, 34, 35
	MΦ	PG release, chemotaxis, lysis of tumor targets	36, 33, 37
	fibroblasts	PG release, collagenase release, growth	9, 12, 38
	synovium	PG release, collagenase release	9
	endothelium	PG release	39
	hepatocytes	secretion of acute-phase reactants, control of plasma metals: copper up, iron and zinc down	3, 40
Tissue catabolism	muscle	proteolysis	41
	osteoclasts	bone resorption	42
	chondrocytes	cartilage breakdown	43
Central nervous system	hypothalamus	fever	1, 44
	[target]?	sleep	45

is affected by IL-1, e.g. both warmth-seeking (during the rise of fever) and drowsiness are induced. It remains an important issue as to whether a single molecular species is responsible for all these actions, or on the other hand, whether a family of closely related molecules or even contaminating materials are involved. Antisera have been produced that react with several of these candidate IL-1s (21, 23, 25); however, this criterion is not decisive, since in most cases the antisera were not highly specific, and monoclonal antibodies have not yet been produced. The final decision awaits results from gene cloning. For the purpose of this discussion we continue to use the term IL-1 for convenience, but we acknowledge that a single molecular entity with all these functional abilities may very well not exist.

While most studies have in the past concentrated on the actions of IL-1 during inflammation, it now appears possible that IL-1 fulfills ongoing roles in normal physiology. Such a "non-inflammatory" role has become plausible with the detection of IL-1 activity in normal serum (59) and urine (17). Although inhibitors occur simultaneously in these fluids and can mask the in vitro detection of serum or urinary IL-1, some in vivo functions, as yet unknown, could be mediated by these basal IL-1 levels. These normal serum levels are observed to vary, with increases occurring during exercise (60) or even during diurnal periods of waking (M. Kluger, personal communication). Do these basal levels of IL-1 serve the organism in some important ongoing way? Will deficiencies lead to pathologies, as with other hormones?

While the significance of basal IL-1 levels is a speculative matter at present, a deficiency in inducible IL-1 levels has been suggested to underlie the common variable immunodeficiency syndrome in one patient (61). On the other hand, excessive IL-1 production is also associated with a variety of pathological states. Rheumatoid arthritis may involve a chronic local excess of IL-1 in the synovia (9, 62). Future efforts will no doubt be directed to controlling the production and action of IL-1 in patients suffering from this crippling disease. The surprising observation that therapy using preparations containing antibodies against HLA-DR (the Ia-equivalent in man) can alleviate symptoms of rheumatoid arthritis (63) may relate to blocking IL-1 production, perhaps based on the proposed role for Ia in triggering IL-1 release (49, 50). Some of the symptoms of gout may arise from the stimulation of IL-1 release by urate crystals (48). Gingivitis is associated with elevated levels of an IL-1–like activity in gingival fluid (64). Another excess–IL-1 disorder may result from ultraviolet irradiation of the skin, which some suggest is a trigger of IL-1 release from keratinocytes (65). It has also been suggested that IL-1 may mediate the muscle catabolism (wasting) that can follow trauma (5, 66).

Immunologic Targets

IL-1 affects several types of cells involved in immune responses, including T cells, B cells, and macrophages. The mitogenic effect on thymocytes was recognized earliest and remains the reference assay for IL-1. This assay is based on the ability of IL-1 to costimulate (with PHA or Con A) the proliferation of mouse thymocytes, which are considerably more reactive to IL-1 than are peripheral T lymphocytes. Why do thymocytes react so strongly to IL-1 relative to peripheral T cells? Perhaps immature T cells are more sensitive to IL-1 than mature cells. On the other hand, both thymic and peripheral T cells may recognize IL-1 but react in different ways; whereas the former react by proliferating, the latter produce lymphokines. Another explanation of the apparent difference in IL-1 sensitivity of thymic and peripheral T cells is that preparations of spleen or lymph-node cells may produce higher endogenous levels of IL-1 than thymocyte preparations. The latter interpretation is supported by the findings that if peripheral lymphoid cell suspensions are depleted of adherent (27) or Ia-positive (28, 49, 50) accessory cells, the resultant depression in T-cell mitogenic responses to lectins (28) or certain antigens (49, 50) can be reversed by IL-1. Thus, when depleted of potential cellular sources of IL-1, peripheral T cells, like thymocytes, can be shown to display considerable proliferative reactivity to IL-1.

The cellular basis of IL-1's action on T cells is shown in Figure 1, which illustrates a view of IL-1's role in the cell-cycle progression of T cells from the resting state (G_0), through G_1, and into cell division (S phase). Several groups propose (27, 28) that IL-1 stimulates T cell division indirectly, by acting as a cofactor along with another stimulus such as antigen or a mitogenic lectin. These combined stimuli then result in the release of IL-2, which in turn induces T cells bearing IL-2 receptors to enter the S phase of the cell cycle. Expression of the IL-2 receptor results from stimuli such as antigen plus Ia or lectin (27, 28), and IL-1 has been observed to augment the expression of the IL-2 receptor (D. Mannel, W. Falk, T. Diamanstein, W. Droege, S. Mizel, personal communication). Moreover, one T-cell clone has been reported that requires IL-1 for expression of the receptor for IL-2 [(67); C. Janeway, personal communication].

T cells depend on accessory cells, such as MΦ, for presentation of antigens and lectins. Is IL-1 an essential component of antigen/lectin presentation, as implied by Figure 1? The tentative answer is that IL-1 is probably required for some T cells and some stimuli, while for other T cells and stimuli, it (at least in secreted form) is probably not. Demonstrations supporting an IL-1 requirement in antigen/lectin presentation are based on

its ability to restore T-cell activation when (*a*) freshly explanted cell populations are depleted of antigen-presenting cells by mechanical methods (27) or anti-Ia treatment (28, 49, 50), or (*b*) when antigen-presenting cells are fixed with paraformaldehyde (68) or exposed to ultraviolet radiation (69). Thus IL-1 restored T-cell activation when antigen-presenting cells were either too few or metabolically impaired. Although these findings are consistent with a requirement for IL-1, they do not prove that IL-1 participates in antigen presentation by normal antigen-presenting cells; the possibility remains that several alternative mechanisms lead to T-cell activation. Most investigators have concluded that, in addition to secretion of IL-1, accessory cells provide other essential functions for T-cell activation; the full spectrum of these additional accessory cell functions is unclear but is generally thought to include catabolism and display of antigens, as well as providing "cell-association" molecules, one of which is Ia.

On the other hand, a number of observations suggest that IL-1 is not required to activate T-T hybrids, despite their dependency on accessory cells displaying appropriate Ia and antigen molecules. This conclusion is founded on the observation that antigen-presenting cells that have been metabolically inactivated by paraformaldehyde (and are presumably

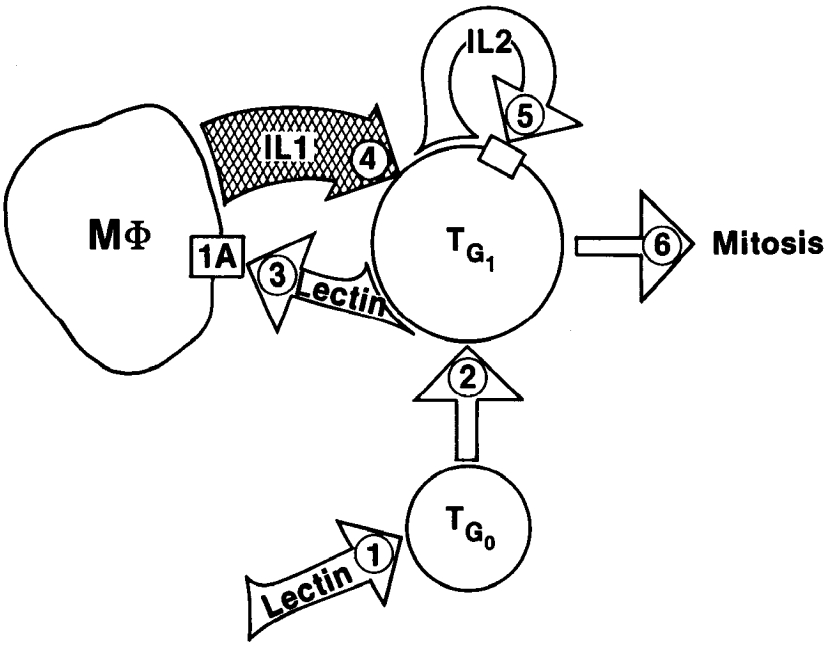

Figure 1 The role of IL-1 in T-cell mitogenesis.

incapable of IL-1 production) are nevertheless adequate to stimulate IL-2 release from T-T hybrids (70). This apparent IL-1–independence of T-T hybrids may not however reflect normal T-cell physiology, since T-cell clones grown with antigen and feeder cells can be stimulated by IL-1 (67, 71), but this IL-1–recognition capacity is frequently lost upon fusion with the BW5147 thymoma cell (71) in the production of T-T hybrids. Another case against a T-cell requirement for IL-1 during presentation has recently been made in analyzing the activation requirements of the L3T4-positive subset of mouse T cells. These T cells can be activated by Con A to secrete IL-2 and express IL-2 receptors. Although accessory cells are required, they can be of diverse types (even T-T hybrids) and need not secrete detectable levels of IL-1 [(72); E. Shevach, personal communication]. Moreover, some transformed cell types have been observed to provide accessory cell function without apparent IL-1 secretion (73).

Recent studies however, suggest a reinterpretation of apparent IL-1–independent accessory-cell function. Paraformaldehyde-fixed MΦ, while incapable of secreting IL-1, have been shown to express a membrane-bound IL-1–like activity (E. Kurt-Jones, D. Beller, S. Mizel, E. Unanue, submitted for publication). This membrane-associated material may permit cells that fail to secrete IL-1 to nevertheless provide an IL-1 signal. The possibility thus exists that IL-1 participates in some or all accessory cell–dependent T-cell activation.

Whether required during antigen presentation or not, IL-1 can produce potent enhancement of immune responses (50), and stimulating its production in vivo may be a common mode of action of immunological adjuvants. In vivo enhancement of immune responses by IL-1 could proceed directly, by stimulating helper T cells (T_H), and could also occur indirectly, through immunoregulatory mechanisms. It has been observed, for example, that temperature elevation, as elicited by IL-1 during fever, can powerfully augment the T_H-activation process (74, 75).

Another immunoregulatory role of IL-1 may be in controlling the balance between T_H and T_S. As shown in Figure 2, IL-1 is an up-regulator for T_H in vitro; it promotes the generation of $Lyt1^+$ T_H (panel A) and inhibits the generation of $Lyt2^+$ T_S (panel B). The latter effect could occur by a direct inhibition of T_S development, or through stimulation of T_H in the cell population which subsequently masks T_S. In addition to favoring the balance of T_H over T_S, IL-1 appears to protect T_H from T_S (panel C) and from $T_S F$ (panel D). These "contrasuppressive" (76) properties of IL-1 could also account for its ability to reverse various states of T-cell unresponsiveness occurring when Ia^+ accessory cells are infrequent or absent. For example, chronic treatment of mice with anti-IA from birth renders T cells unresponsive in the T-cell proliferation assay, but IL-1 can restore

proliferation (50). IL-1 also reverses T-cell unresponsiveness in the Ir gene–controlled response to GAT. GAT fails to prime for T-cell proliferation in mice of the $H2^q$ haplotype (77) and can be shown to prime T_S (78) preferentially; this unresponsiveness to GAT is controlled by IA molecules. Exogenously supplied IL-1 was observed to permit T cell–proliferative responses to GAT in nonresponder DBA/1 mice (50). One explanation of IL-1 restorative powers in genetic nonresponders, or anti-IA-treated mice may be that T_H/T_S balance is altered, perhaps much as we observed using the in vitro systems as shown in Figure 2. We have also suggested that one role for Ia in IL-1–producing cells may be to receive stimulatory signals from T cells. Thus, genetic unresponsiveness to certain antigens may relate to defects in T-to-MΦ signalling (49), reflected in an inability to produce IL-1. Whether the unresponsiveness to GAT is actually the result of insufficient IL-1 or not, the T cell–proliferative response is powerfully augmented by IL-1, as are defective proliferative responses resulting from acute or chronic depletion of IA^+ cells. These findings encourage the use of IL-1 as a

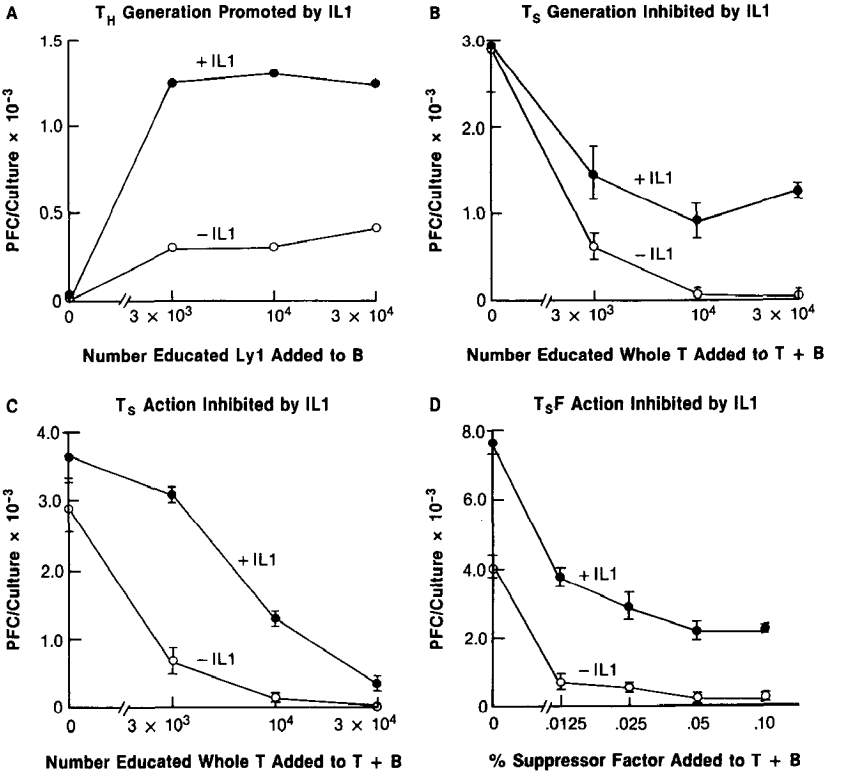

Figure 2 IL-1 regulates the balance of T-cell help versus suppression [for methods see (75)].

potential therapeutic in cases of flagging or actively suppressed immunity.

Other cells of the immune system in addition to T cells can respond to IL-1. B lymphocytes are reactive to IL-1 during at least two stages of development: first, at the B-cell precursor stage; second, following antigen stimulation. Initially, an uncharacterized macrophage factor was reported to induce maturation in precursor B cells (79). Using different criteria for maturation of pre–B cells, recent findings have identified IL-1 as the maturation-inducing activity in MΦ supernatants (80). IL-1–driven maturation has also been examined using a cell line with pre–B cell characteristics; this line, like pre–B cells, produces cytoplasmic μ chains, and fails to synthesize light chains or express membrane immunoglobulin. Purified IL-1 was observed to stimulate κ light-chain production and subsequent membrane immunoglobulin expression by this pre–B cell line (80). The second stage at which B cells are sensitive to IL-1 occurs during antigenic stimulation; MΦ supernatants have long been known to promote these B-cell responses (29, 30). Using several methods for stimulating B-cell responses, a number of laboratories have now concluded that IL-1 acts in synergy with other lymphokines in promoting B-cell proliferation (30, 31, 81). Figure 3 depicts the sequential signals that activate B cells and propel

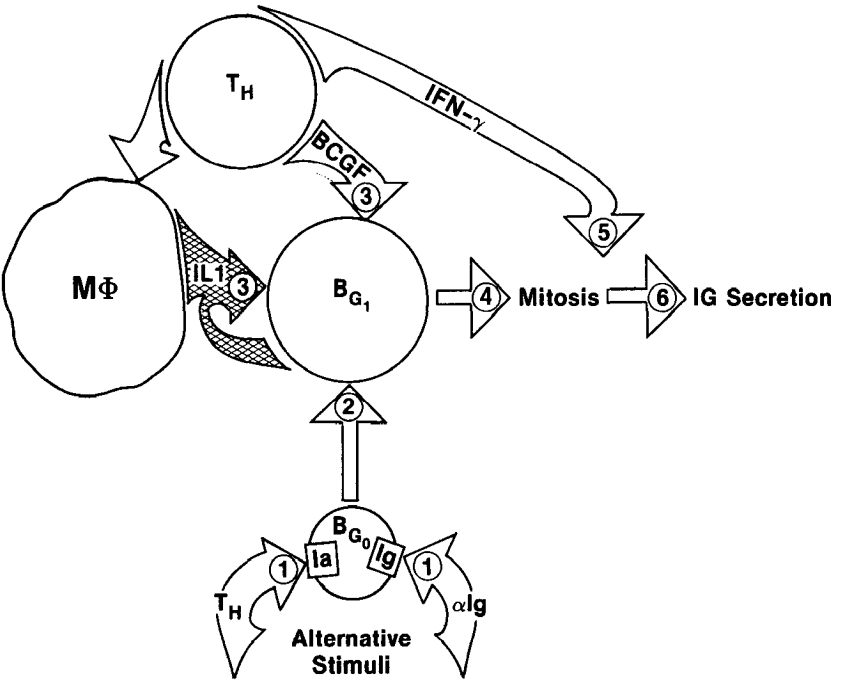

Figure 3 The role of IL-1 in B-cell mitogenesis and differentiation.

them through cell division and Ig secretion. Resting B cells (G_0 stage) can be driven into cycle by several stimuli, though the type of stimulus may depend on the B-cell subset (82). One such stimulus can be provided by T_H via recognition of antigen plus Ia on the B cell [(83); reviewed in (31, 82)]: Another type of stimulus may be via a lymphokine (which may also participate in the signal from T_H) (84), and yet a third type of activation can be achieved through extensive cross-linking of membrane Ig (85). B-cell growth factor is required early in the sequence of stimuli (31). IL-1 also acts relatively early (within the first 24 hr) following stimulation by antigen or anti-Ig (30, 31), and, along with B-cell growth factor and possibly IL-2 (86), drives B cells through mitosis. Late-acting lymphokines, possibly including IFN-γ (87, 88), then promote differentiation to Ig secretion. During immune responses the source of IL-1 for B-cell growth may be macrophages stimulated by T_H. However, recent findings suggest that activated B cells can themselves produce IL-1–like factors (89), and this may account for the observation that at high cell density, B cells required no exogenously added IL-1. This apparent lack of IL-1 requirement at high cell density was previously assumed to represent low-level contamination by MΦ (31).

The macrophage itself is the third cell type within the immune system that responds to IL-1. This response consists of chemoattraction (33), PGE_2 release (36), and activation for tumor-cell killing (37). It remains to be established whether the same MΦ cell that produces IL-1 can also respond to it by PGE_2 release, or whether the production of IL-1 and PGE_2 are achieved by distinct MΦ subsets. In either case, the PGE_2 produced at a local site by MΦ responding to IL-1 can inhibit subsequent IL-1 production (K. Matsushima, personal communication) concomitant with reduced expression of Ia (90).

The Target Membrane and Beneath

Since peptide hormones generally interact with target cells via high-affinity membrane receptors, IL-1 may be expected to do so as well. Such studies have been limited by the quantities of purified IL-1 available for analyzing binding to target-cell membranes but should progress when large quantities of IL-1 are produced by gene cloning.

Do the multitude of IL-1 actions on various cell targets involve identical receptor units? Two types of indirect evidence already suggest that IL-1 receptors on different cell types may be distinct, based on studies suggesting that separate sites on the IL-1 molecule may trigger different types of target cells. The first intimation that fever induction and thymocyte mitogenesis might involve distinct sites on IL-1 molecules (implying different target-cell receptors) comes from reports that IL-1 produced by stimulation with certain forms of muramyl dipeptide lacks pyrogenicity (91, 92), suggesting that this stimulant, as compared to LPS, stimulates production of different

species of IL-1. However, since the "IL-1 activity" detected in these supernatants by thymocyte bioassay was not characterized biochemically, the detected IL-1 activity could be completely unrelated to conventionally produced IL-1 molecules [others have in fact reported non–IL-1 molecules mimicking the mitogenic action of IL-1 on T cells, e.g. carboxypeptidase B and, to a lesser extent, serine esterases (93)]. A second case for multiple species of IL-1 receptors could be built on the assumption that if different sites on the IL-1 molecule engaged distinct types of receptors on different target cells, then certain peptide fragments of IL-1 might be expected to display some biological activities but not others. Such a fragmentation may occur naturally; in a recent report, peptides of 2 and 4 kilodaltons with IL-1–like activities were discovered in human urine (17). These peptides could represent breakdown products of the native 15 kilodalton IL-1 molecule; one of these urinary peptides (2 kilodaltons) reacted with thymocytes but not with fibroblasts, suggesting that separate sites on the IL-1 molecule might act on fibroblasts versus thymocytes.

Little is known concerning postreceptor events, but IL-1 appears to induce the breakdown of arachidonate in several cell types. Pharmacological inhibitors of the cyclooxygenase arm of arachidonate metabolism, such as aspirin and indomethacin, have long been known to inhibit the pyrogenic action of IL-1 [reviewed in (94)] and, more recently, were shown to block the ability of IL-1 to stimulate PGE release in vitro from hypothalamic cells (fever center) (44). Production of PGE in response to IL-1 occurs rapidly (within 30 min) in hypothalamic and other brain tissue and apparently depends on protein synthesis since cycloheximide blocks PGE production stimulated by IL-1 on brain tissue (44). IL-1 also stimulates PGE production by MΦ (36), and prostaglandins probably occur as intermediaries in the stimulation of muscle proteolysis by IL-1 (41). The action of IL-1 on T cells may also involve the production of arachidonate breakdown products, possibly leukotrienes, since an inhibitor of the lipoxygenase pathway partially inhibits this process (36). Hence, IL-1 may have the general effect of stimulating arachidonate metabolism in its target cells.

MODULATORS OF IL-1 PRODUCTION AND ACTION

We have described above how IL-1 is produced and how it acts. We now consider methods of influencing these processes, i.e. enhancers and inhibitors of IL-1 production and action. It is important to understand how IL-1 production and action can be modulated for several reasons; first, to appreciate the natural regulation process; second, to probe the biochemical mechanisms of production and action through the use of pharmacologic

agents; and third, to provide therapeutic approaches yielding enhancement of IL-1 activity when patient immunity is deficient, and diminution of IL-1 activity when it contributes to the pathology of chronic inflammation.

Interferons may represent natural up-regulators of IL-1 production, since both IFN-α (95) and IFN-γ (unpublished results) augment IL-1 release. Since serum generally promotes IL-1 production, cofactors may exist, but these are uncharacterized as yet. The calcium ionophore A23187 strongly enhances IL-1 release (52), indicating a role for intracellular calcium; and hydroxyurea, which may arrest cells in the G_1 stage of the cell cycle, is also a powerful promoter of IL-1 release (K. Matsushima, unpublished results). All these enhancers must act in conjunction with a stimulus for IL-1 release, such as LPS or silica. Inhibition of IL-1 production by monocytes can of course be achieved by many agents that disrupt normal cell function, or by inhibitors of mRNA or protein synthesis; however, several reported inhibitors are more revealing. For example, corticosteroids, which are well known for their immunosuppressive and antiinflammatory properties, block IL-1 production (96). The mechanism by which steroids interfere with IL-1 production is presently unknown, but it could involve their established ability to inhibit arachidonate breakdown. Arachidonate metabolism in IL-1 production is implicated further by the ability of inhibitors of lipoxygenase to inhibit IL-1 production (54), suggesting a role for leukotrienes in IL-1 production. On the other hand, PGE_2, another arachidonate metabolite, inhibits IL-1 production and may participate in negative feedback control of IL-1 production, since IL-1 is known to stimulate PGE production by MΦ and other cells. Cyclosporin, an immunosuppressant, is reported to block T cell–induced, but not LPS-induced IL-1 production (97). Aging of MΦ in vitro reduces the cells' ability to produce IL-1 (98). Exposing MΦ to ultraviolet radiation is yet another method reported to inhibit IL-1 production (69) and may account for the impaired immunity in mice so treated.

Many of the modulators of IL-1 production that we have discussed also affect membrane Ia levels. IFN increases Ia (90) as well as IL-1, whereas steroids (96), aging (99), and PGE_2 (90) reduce both Ia and IL-1; and ultraviolet irradiation both alters Ia shedding (100) and reduces IL-1 production. Thus Ia and IL-1 may be coordinately regulated. Both may be produced simultaneously; alternatively, Ia molecules may be produced first, then subsequently are required to participate in receiving the stimulatory signals leading to IL-1 release (49). Consistent with the latter notion is the proposal that cyclosporin blocks the ability of T cells to recognize Ia (101), which would account for its inhibition of T cell–induced, as distinct from LPS-induced, IL-1 production.

The action of IL-1 on T cells can also be modulated by a number of agents, most of which are inhibitors. Since the observed mitogenic effect of IL-1 on thymocytes is presumed to be mediated by IL-2 (102), any agent that affects IL-2 production or action would of course affect the thymocyte assay. The calcium ionophore A23187 is a powerful augmenter of IL-1's action on thymocytes (52), implying a role for intracellular calcium; inhibitors of lipoxygenase diminish IL-1's action (36), suggesting a role for leukotrienes; but it is unknown as yet whether the site of action of the ionophore and the lipoxygenase inhibitors is at the level of production and/or at the level of action of IL-2. Several agents have been described that interfere with IL-1 at the level of IL-2 production and do not block the action of IL-2; these agents include hydrocortisone (103), proteins isolated from human urine (17, 104), and products released from some tumor cells (105). The urinary IL-1 inhibitor may be related to naturally occurring enzyme inhibitors present in serum; moreover, this inhibitor may perform a physiological role in controlling the action of IL-1, since it is observed to rise during fever (104), a time at which circulating IL-1 would also be present at elevated levels. PGE_2, another inhibitor of the thymocyte assay, is reported to block IL-2 production (106). Cyclosporin is a clinically employed immunosuppressant that inhibits the thymocyte assay (107), probably by blocking both IL-2 production and action (108). One action of cyclosporin may be to impair the ability of T cells to acquire sensitivity to IL-1, since it inhibits the IL-2–stimulating effect of IL-1 added early in the MLR, but not late (after T cells have become IL-1 sensitive) (109). Consistent with this view, another study observed that cyclosporin did not block the direct mitogenic action of IL-1 on thymocytes (which may be mediated by cells already sensitive to IL-1), but did impair the ability of IL-1 to augment Con A mitogenesis (which could involve Con A–driven acquisition of sensitivity to IL-1) (107). It remains to be clearly established whether other natural suppressor mechanisms, such as TsF, are active on IL-1 production or action, although data shown in Figure 2 suggest that IL-1 counteracts the generation and action of T suppressor cells.

Thus, a growing number of modulators are being described that function at the level of either production or action of IL-1. These agents are valuable tools to the biologist and clinician, and we can anticipate further interest in their development and study.

CONCLUSION

IL-1, a hormone-like peptide produced by activated MΦ, exerts a number of actions on cells of the immune system. The cellular targets include T cells, B cells, and MΦ, and the biological functions induced encompass a variety

of growth and differentiation processes. Many cell types other than MΦ can produce IL-1–like factors, and the actions of IL-1 apparently extend beyond the immune system and may provide the unifying principle for a number of inflammatory processes. However, until the degree of molecular similarity among "IL-1–like factors" is clarified, and until the multitude of "IL-1 actions" is unambiguously ascribed to a single molecular entity, we would do well to recall the words of the late physicist Wolfgang Pauli: "Let no man join together what God hath put asunder." Nevertheless, we are faced with the exciting possibility that IL-1 forms a major link between the immune system and the rest of the body—a bidirectional link allowing immune responses (which generate IL-1) to influence the organism at large, and conversely, permitting other tissues (which also produce IL-1) to influence the immune system.

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CONTENTS

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IMMUNOLOGY OF INSULIN-DEPENDENT DIABETES MELLITUS

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INTRODUCTION

Polyuria, polydipsia, polyphagia, and mellituria have been recognized since antiquity as the classic symptoms of diabetes mellitus. Research has shown, however, that diabetes is not a single disease but a group of disorders having in common hyperglycemia. In addition to its effects on carbohydrate metabolism, diabetes also affects the metabolism of protein and fat and commonly leads to serious complications affecting many organ systems. In its various forms, diabetes afflicts about 5% of the population in most Western societies (1).

Studies of the natural history and pathogenesis of hyperglycemia, together with recent advances in biotechnology, have led to the system of classification developed by the National Diabetes Data Group (2). Among the various diabetic syndromes now recognized, insulin-dependent diabetes mellitus (IDDM or Type 1 diabetes) and noninsulin-dependent diabetes mellitus (NIDDM or Type 2 diabetes) account for the great majority (>90%) of patients with the disease. The principal features that distinguish IDDM and NIDDM are summarized in Table 1. In this review we shall focus on advances in the study of IDDM, a syndrome believed to have an autoimmune pathogenesis.

HUMAN INSULIN-DEPENDENT DIABETES MELLITUS

All patients with IDDM share a common clinical finding: dependence on insulin for survival. This results from the near total disappearance of the

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Table 1 Classification of diabetes mellitus

	Insulin-dependent (IDDM; Type 1)	Non insulin-dependent (NIDDM; Type 2)
Age at onset:	Usually under 25	Usually over 40
Prevalence:	0.2–0.5%	2–4%
Sex distribution:	Male = Female	Female preponderance
Clinical onset:	Acute to subacute; ketosis may be present	Chronic nonspecific symptoms
Insulitis:	Present	Absent
Peripheral lymphocyte abnormalities:	Present	Absent
Association with other autoimmune diseases:	Yes	No
Genetics:	Frequently familial; strong association with histocompatibility markers HLA-Dr 3/4	Very frequently familial; no association with histocompatibility markers
Insulin treatment:	Always necessary	Not always necessary; patients may respond to diet and/or oral agents
Former terminology:	Juvenile onset diabetes Ketosis-prone diabetes Brittle diabetes	Adult onset diabetes Ketosis-resistant diabetes Maturity onset diabetes of the young (MODY)

insulin-producing beta cells normally found in the pancreatic islets of Langerhans. It is thought that a variety of etiologic factors may lead to this common clinical and pathologic picture. Viruses, stress, environmental toxins, and various immune system alterations have been proposed, but there currently exist no methods for implicating any specific factor in a given patient. Those afflicted with the disease develop a lifelong dependence on insulin injections for survival. This therapy now prevents the deaths from diabetic ketoacidosis that were formerly inevitable, but insulin injections do not prevent the late complications of IDDM. These include blindness, renal failure, neuropathy, and peripheral and coronary vascular disease (3). At present, there is no accepted, safe way in which to prevent the disease or to alter its course once it has begun (4).

EPIDEMIOLOGY

Epidemiologic studies indicate that the incidence of IDDM varies between 7.7 and 30 per 100,000 population per year (5). Japanese, Indians, Chinese, American Indians, Eskimos, South African Blacks, and Polynesians have a relatively low incidence. The highest incidence—over 30 per 100,000 per year—has been reported in Finland. In other Scandinavian countries the yearly rate varies from 15 to 20 per 100,000 (6). Most studies report that both sexes are equally affected (4), though a few have shown a slight preponderance (12–20%) of males among younger diabetic children (7–9). The peak age of onset is 11–12 years of age. A secondary peak occurs at 5–8 years of age (10).

Seasonal variation in the onset of IDDM has been noted. The greatest number of new cases occur in autumn and winter and the fewest in summer (11). This seasonal variation suggests that viral infection may be a precipitating factor in the pathogenesis of the disease (12).

VIRUSES AND IDDM

The association of viral infection and IDDM has been recognized for many years (13). In 1864, a Norwegian physician reported a case of diabetes that developed soon after mumps. A number of studies have since confirmed the temporal relationship between IDDM and antecedant viral infection (14). Among the most elegant of these studies was that of Gamble et al (15). They found that patients with IDDM of recent onset had high titers of neutralizing antibody to Coxsackie B-4 virus when compared either to normal subjects or to patients with IDDM of greater than three months duration. They also demonstrated that the seasonal incidence of diabetes correlated with the annual prevalence of Coxsackie virus infection (12).

IDDM has been reported in up to 20% of patients with congenital rubella (16, 17). An equal number develop impaired glucose tolerance (18). More than one third of congenital rubella patients have circulating islet antibodies (19). Experimental rubella infection in rabbits can cause morphologic changes in the pancreas (20).

Encephalomyocarditis virus–neutralizing antibody has been found in 12% of patients with IDDM compared to 6% of a control population (21). This virus is beta-cell cytotropic (22). Cytomegalovirus, infectious mononucleosis, varicella, polio, influenza, and tick-borne encephalitis have also been temporally associated with IDDM (14). Autopsy study of children who died of various viral illnesses has demonstrated pancreatic insulitis in the absence of ante mortem hyperglycemia (23).

The most convincing evidence of a possible relationship between viral infection and IDDM was reported by Yoon and his coworkers (24). They obtained at autopsy the pancreas from a child who died of severe ketoacidosis soon after the acute onset of IDDM. A prodromal syndrome suggestive of a viral infection had preceded the diabetes. The investigators were able to culture Coxsackie B-4 virus from the pancreas. When administered to a susceptible strain of mice, the cultured virus induced diabetes. There is also an interesting case report of a child from a family without clinically evident autoimmune disease or any history of diabetes who had islet-cell antibodies and viral antibody titers to Coxsackie B-4 virus several years prior to the onset of IDDM (25).

The most popular theories of the pathogenesis of viral diabetes include direct beta-cell damage, immune cytotoxicity directed against beta cells harboring a foreign viral antigen, and the induction of chronic autoimmunity directed against beta cells. Given appropriate genetic susceptibility in any of these circumstances, the host becomes diabetic. An alternate theory is suggested by a recent study showing that autoantibodies to various endocrine target tissues can be induced by injecting neonatal animals with rheoviruses (26). There remains, however, no conclusive demonstration that IDDM is due to a viral infection. While it is reasonable to believe that viruses are involved in the pathogenesis of some cases of IDDM, considerable additional study will be needed to determine the relative importance of viruses in the disease process.

GENETICS

IDDM is a heritable disorder, but the exact mode of transmission is unclear (27). The overall risk is 1 in 300–400 for white children (28, 29). If one parent has IDDM, the risk is about 8–10% for their offspring. If both parents have the disease, the risk is over 23% (30). IDDM may be more common in the offspring of diabetic fathers than those of diabetic mothers (31). The youngest child in a family appears to be most susceptible to the disease (32). Many studies have demonstrated familial aggregation, and twin studies also support this contention. Between 30 and 50% of monozygotic twins are concordant for IDDM (33, 34).

Almost all modes of inheritance have been suggested at various times, but evidence does not conclusively support any one hypothesis. A single-gene model would not account for the heterogeneity of IDDM, and various polygenetic models have been proposed (35, 36). Diabetes has been linked to the Kidd blood group located on chromosome 2 (37) as well as to other nonhistocompatability (HLA) markers (38).

The most powerful insights into the genetics of IDDM have come from

the study of histocompatibility genes. The association between IDDM and HLA antigens has been known for over a decade (39–41). The first association recognized was with certain alleles at the B locus (B8 and B15) on chromosome 6. More recent studies suggest that susceptibility to IDDM is associated primarily with genes at or near the HLA-D/Dr region (42–44). Family studies have demonstrated that more than 90% of Caucasian subjects with IDDM are positive for HLA-Dr3 and/or 4 haplotypes (45, 46). Population studies indicate that the risk of becoming diabetic is 1 to 3% if a subject has HLA-Dr3 and -Dr4 haplotypes (47, 48). It has been estimated that the risk of IDDM in children who share the HLA-D haplotype of a diabetic sibling may be as high as one in four (19). Individuals with HLA-B7 or Dr2 seem to be protected from the disease, but this may reflect only linkage disequilibrium (49).

Concordance of IDDM in identical twins varies between 30 and 50% (33, 34, 50). The majority of concordant twins are HLA-Dr3 and/or HLD-Dr4, while disconcordant identical twins have neither antigen (51). The effects of both HLA-Dr3 and HLA-Dr4 may be additive. There is reported to be 70% concordance if twins have both Dr3 and Dr4 antigens, as contrasted with 40% concordance if twins have either HLD-Dr3 or -Dr4 but not both (51). It may be that there exists a diabetes susceptibility gene located within the HLA complex in association with the HLA-Dr3 and -Dr4 region.

Dr gene products are class II antigens present on the plasma membrane of cells. They are composed of an alpha, or heavy, chain (M_r 34,000) and a beta, or light, chain (M_r 29,000) (52). These antigens (the Ir gene products) are involved in immune regulation. Besides the HLA-Dr antigens, other class II antigens have recently been identified. HLA-DC, which is in linkage disequilibrium with HLA-Dr, is associated with IDDM while HLA-SB is not (53, 54).

The HLA-D region has recently been studied in more detail using recombinant DNA technology. DNA probes and genomic blotting techniques demonstrate that differences exist in the size of fragments generated by restriction endonuclease treatment of Ir DNA. This technology has been used to define five to six alpha genes (one Dr alpha, three or four DC alpha related, and one SB alpha) (55). There are also seven beta genes (three Dr beta, two DC beta and two SB beta). HLA-DC beta-chain sequences are more strongly associated with IDDM than are HLA-Dr antigens (56). Additional analysis of flanking genes will be required to determine the exact class II antigens most strongly associated with IDDM.

Although a predisposition to IDDM may indeed be genetically determined, there could be individuals with diabetes in whom nongenetic factors are equally or more important. Viral infections or toxins may be obligate cofactors in the pathogenetic process.

IMMUNOLOGIC FACTORS

A growing body of data suggests that human IDDM is an autoimmune disease. The data come from studies of morphology, cellular immunity, and humoral immunity, as well as from clinical observations and clinical trials of immunosuppressive therapy.

Morphological Studies

Autopsy specimens of the pancreas from subjects who died shortly after the onset of IDDM have shown a specific lesion, insulitis. This term was coined by Von Meyenburg to describe an infiltration of inflammatory mononuclear cells into the islets of Langerhans (57). In one study, islets from 16 of 23 patients with recent onset IDDM revealed insulitis (58). In another study, 6 of 11 specimens from patients who died within two months of the onset of IDDM revealed the same pathological finding (59). Immunofluorescence study of the cells infiltrating the pancreas of a child with recent-onset IDDM showed that most were cytotoxic/suppressor cells and to a lesser degree T-helper cells (60). Ninety percent of the infiltrating T lymphocytes expressed HLA-Dr antigens.

Other studies, however, have found insulitis rarely or not at all (61). This discrepancy may be related both to the time interval between the onset of diabetes and death and to the clinical circumstances under which the diagnosis became apparent. At the time IDDM is clinically obvious, patients have already lost approximately 90% of their beta cells (62). The remaining islet-cell types appear to be present in normal numbers (63).

It is reasonable to assume that the destructive autoimmune process of IDDM may occur over a prolonged period, and only when a sufficient number of beta cells are destroyed does clinical evidence of IDDM appear. The time at which diabetes is detected, and its clinical severity, may also depend on many factors. For example, stress or a viral infection increases the physiological need for insulin and places increased demands on beta cells. The appearance of diabetic symptoms is thus a complex function of the extent and duration of the process that is damaging beta cells and of various precipitating factors (Figure 1). In a case precipitated by stress, sufficient beta cells may remain at the time of death to serve as the antigenic target for the autoimmune pathologic process, while another unstressed individual may be diagnosed when few if any beta cells survive and the destructive process has subsided. The presence or absence of insulitis will thus depend on the number of remaining beta cells and/or the duration of the process.

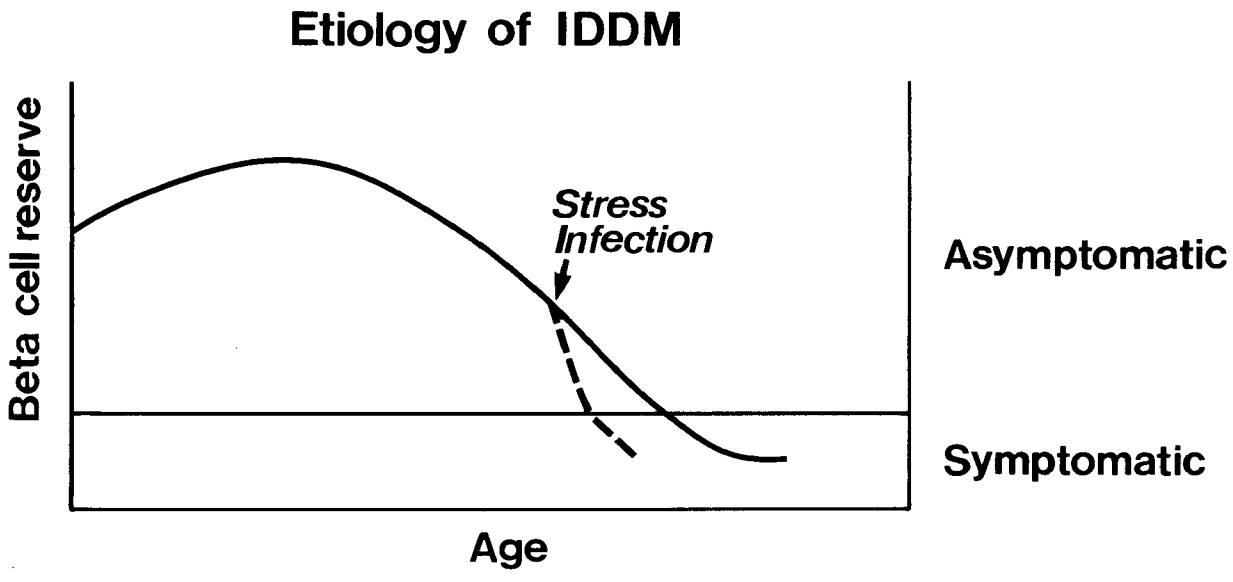

Figure 1 Schematic representation of the relationship between beta cell reserve and the clinical onset of IDDM. Beta cell reserve may be regarded as a function of beta cell number and the functional capacity of remaining beta cells to secrete insulin in response to glucose challenge. In some instances (solid line) the indolent process of immune beta cell destruction may not lead to diabetes until most cells are destroyed and there is insufficient insulin to meet even the most minimal homeostatic requirements. In other instances (dashed line), stress or infection may supervene and accelerate the loss of beta cells numbers and/or function, while at the same time producing a state of enhanced insulin resistance. Symptomatic diabetes may then be observed at an earlier point in the autoimmune process. Resolution of stress may result in a return of some beta cell reserve and the "honeymoon" period often observed soon after the diagnosis of diabetes. Eventually, however, the autoimmune process leads inevitably to permanent diabetes.

Cellular Immunity

CIRCULATING LYMPHOCYTES Studies of circulating lymphocytes in IDDM have yielded variable results. Normal T- and B-lymphocyte numbers have been found in the circulation of subjects with IDDM, with the numbers being independent of the degree control of diabetes (64, 65). Other studies, however, report decreases in T-lymphocyte numbers that were either dependent on (66) or independent of (67) the degree of glycemic control.

Monoclonal antibody studies of lymphocyte subsets have provided interesting findings. Helper/inducer lymphocytes (OKT_4, Leu 3A) have consistently been found to be unchanged when compared with controls. However, suppressor/cytotoxic subsets (OKT_8, UCHT-14) are variously reported to be normal, increased, or decreased when compared to nondiabetic control populations (68). In one study, the OKT_4/OKT_8 ratio was found to be increased in acute (<2 months) IDDM and then normalized with time (69).

A reduced proportion of high-affinity, E rosette–forming cells has been found in IDDM, while total T lymphocytes were normal (67). Circulating Ia^+ T cells have been found in increased numbers in acute IDDM (70–71). These cells are presumptively the activated T cells that are present in many immunologic conditions (72). Another study of patients with IDDM has demonstrated a decrease in T lymphocytes with IgG Fc receptors (T-gamma cells) (73).

Although B-lymphocyte numbers are usually normal, increased numbers of B lymphocytes have been found when islet cell–surface antibodies were also present in the serum (74). Furthermore, increased immunoglobulin producing–B lymphocytes have been noted in the blood of patients with IDDM, suggesting the presence of activated, antibody-producing B lymphocytes (69).

The conflicting results of these studies of lymphocytes in IDDM probably reflect the limitations inherent in monoclonal antibody techniques when applied to patients with variable clinical and immunologic status at the time of study. The subsets defined in this way may not accurately define the diabetic state and could contain subpopulations of much greater importance. Furthermore, cells found in the peripheral circulation may not reflect the pathologic processes occurring in the islet. More detailed studies may clarify these findings in the future.

A recent report describes five patients with newly diagnosed IDDM in whom peripheral lymphocytes were isolated, tagged with $indium^{111}$ and reinjected into the same recipient (75). Three of the five patients given autologous radiolabeled lymphocytes appeared to show localization of activity in the region of the pancreas, as detected by emission-computerized

scanning. This potentially important study, which has not yet been confirmed, suggests that migration of lymphocytes to the islets occurs in IDDM and may be detected noninvasively.

STUDIES OF LYMPHOCYTE FUNCTION One of the first functional abnormalities of cell-mediated immunity in IDDM was detected with the leukocyte migration test. These studies suggested that leukocytes of patients with IDDM are sensitized to pancreatic antigens, causing inhibition of migration (76–78). Another significant report indicated that lymphocytes (obtained from acutely diabetic children) that are cocultured in vitro with human insulinoma cells will kill the tumor cells (79). Unfortunately, this human insulinoma cell line is no longer available and the report lacks confirmation. Lymphocytes from a patient with acute IDDM transferred passively to athymic mice cause a slight rise of blood glucose in the recipients (80); however, other laboratories have been unable to confirm this finding (81).

Suppressor–T cell activity is reportedly defective when lymphocytes from patients are stimulated either by the mitogen concanavalin A (con A) or by pancreatic antigens (82–84). In one study, suppressor-cell activity was related to disease duration, while in others no relationship was evident. The abnormality of suppressor cell activity may be related to a paucity of suppressor T lymphocytes, as suggested by the decrease in OKT_8-labeled cells in the patients studied (85). Abnormal suppressor T-lymphocyte function has also been demonstrated in nondiabetic subjects who are HLA-Dr3 and/or Dr4 positive; this suggests an immune regulation abnormality related to the genetic background (86–88). Phytohemagglutinin responsiveness of lymphocytes in IDDM is depressed, especially in poorly controlled diabetes (89, 90).

When peripheral blood lymphocytes are obtained from IDDM subjects and incubated with mouse islets, they reportedly inhibit insulin secretion when the islets are perturbated with either glucose or theophylline (91, 92). Another defect of lymphocyte function has been found using the autologous, mixed lymphocyte reaction (AMLR). In the absence of added antigens, autologous non–T cells proliferate. An abnormal AMLR has been found in patients with IDDM that did not correlate with either duration or glycemic control (93). Recent studies suggest that the defect in the AMLR may be due to a lack of interleukin-2 (IL-2) production (94). Complement-mediated, as well antibody-dependent, cellular cytoxic activities are increased in the serum of patients with IDDM (94, 95).

LYMPHOKINE PRODUCTION Interferon production is decreased in IDDM (96). Recent studies have also demonstrated defective production of IL-2 in IDDM (97–98). This defect does not appear to be related to the adequacy of

metabolic control. The possibility that a cytokine needed to modulate or suppress immunocyte activity may be deficient in IDDM deserves further study.

Humoral Immunity

The presence of autoantibodies in the serum of patients with autoimmune endocrine diseases has been recognized for some time. Islet-cell autoantibodies in IDDM were first reported by Bottazzo et al (99). Since the original description, a number of assay systems have revealed several classes of islet-cell antibodies (100–102).

ISLET CELL CYTOPLASMIC ANTIBODIES (ICA) Using undiluted serum, islet cell–cytoplasmic antibodies (ICA) can be detected by indirect immunofluorescence using cryostat sections of fresh post mortem human pancreas from blood group O donors (103, 104). Serums from IDDM patients usually cross-react with the cytoplasm not only of beta cells but of all endocrine pancreatic cell types. The antibodies are principally of the IgG class. ICA are found in 0.5% of the normal population and in approximately 70% of cases of newly diagnosed IDDM (105, 106). The prevalence of ICA among subjects with other autoimmune endocrine diseases without clinical evidence of diabetes is 6% (107). The frequency of ICA decreases with the duration of IDDM, and ICA are infrequent in cases of many years duration (108).

An alternative procedure for the detection of ICA uses pancreata that have been fixed in Bouin's solution (109). Although this method is technically easier, it is unreliable and is not recommended (110).

Adding complement to the standard immunofluorescence sandwich technique seems to increase the sensitivity of the method (111). It has been shown that up to 22% of serums that are ICA negative by other techniques are positive when retested using this refinement. Complement-fixing, islet-cell antibodies (CF-ICA) may be better markers for islet damage since they occur more frequently at the onset of diabetes and disappear more rapidly from the serum during the subsequent clinical course than do ICA.

Islet-cell antibodies have been used to predict the clinical onset of IDDM. Some glucose-intolerant subjects and diabetics using oral hypoglycemic agents who had positive ICA have later become insulin-dependent diabetics (108). Studies of the families of patients with IDDM demonstrate that ICA are present years before the onset of clinical diabetes (112). In one study, ICA were present eight years before the onset of IDDM (113). The appearance of ICA has been correlated with deterioration of insulin secretion, but ICA may be present in subjects with entirely normal carbohydrate metabolism (114). It is important to note, furthermore, that ICA may appear and disappear in nondiabetic individuals (115).

Considerable additional study will be necessary before ICA can be regarded as markers of impending IDDM. Some authorities believe that ICA are more likely to be the result of beta-cell destruction than the actual agents of beta cytotoxicity.

ISLET CELL SURFACE ANTIBODIES Other antibodies found in the serums of patients with IDDM appear to be directed against the plasma membrane or surface of islet cells (116). They are identified by indirect immunofluorescence using either fixed islet cells or viable suspensions of rat insulinoma cells, rat islet cells, or mouse islet cells. These antibodies can be quantitated by the use of ^{125}I-labeled protein A or by cytofluorometry (117, 118).

Islet cell–surface antibodies (ICSA) have been found in a third of patients who have had IDDM less than two years (119). ICA and ICSA do not always correlate (120), but both appear to be present during the acute diabetic state (121). ICSA specific to the beta cell are reported to inhibit insulin secretion and to mediate beta cell destruction (102).

Recently, it has been shown that ICSA recognize a 64-kilodalton human–islet cell protein (122). ICSA directed against this antigen were observed two years before the onset of diabetes in the discordant twin of a diabetic who did not have detectable islet cell cytoplasmic antibodies. This 64-kd determinant may represent a major target antigen involved in the pathogenesis of IDDM.

OTHER ANTIBODIES Cytotoxic antibodies directed against islet cells have been detected in a complement-dependent, cytotoxic, islet-cell antibody assay (123) and, as noted above, in antibody-dependent cellular cytotoxicity assays (94). The latter test supports the observation that lymphocytes and serum from newly diagnosed IDDM are both cytotoxic (79). The observation of cytotoxic antibodies assumes added importance given that natural killer (NK) cells have been found to be normal or slightly increased in subjects with IDDM (124). These NK cells may attack other cells, perhaps islets, after interacting with antibody bound to the cell surface. Antilymphocyte antibodies have also been demonstrated in subjects with IDDM (125).

Immunoprecipitating antibodies have recently been found in the serums of newly diagnosed diabetics (102). The antigens used to detect the antibodies were from human cadaver islets from an HLA-Dr3 positive patient. The authors suggest that since class II antigens are not known to be expressed on islets, these antibodies precipitable from diabetic serums may detect a Dr3-linked antigen. Monoclonal antibodies have been produced that can identify islets and can be used as controls in assays for anti-islet antibodies (126). Immune complexes are also present in the serums of patients with acute onset of insulin-dependent diabetes (127). The significance of these complexes is uncertain.

None of the currently available procedures for the assay of islet antibodies can be regarded as routine. The assays require subjective quantification and stringent quality control. Standardization among the various research laboratories is still lacking.

Clinical Observations

An indirect but suggestive piece of information regarding the pathogenesis of IDDM is its association with other autoimmune endocrine syndromes including Addison's disease, rheumatoid arthritis, and Hashimoto's thyroiditis (128). Clinical studies provide another important observation, when diabetic monozygotic twins received a pancreas transplant from nonconcordant, nondiabetic siblings. Sutherland et al reported that insulitis and diabetes recur in the transplanted normal islets (129). This suggests that long after the onset of diabetes the immune system remains capable of destroying islet tissue if the proper antigenic determinant (i.e. the beta cell) is again presented to the host system. Taken together, these observations strongly suggest an autoimmune pathogenesis of human insulin-dependent diabetes. The evidence suggests further that autoimmune destruction of the beta cells may be an indolent, progressive process.

Immunosuppression

Acceptance of the autoimmune hypothesis of IDDM has led clinical investigators from several countries to attempt to reverse the acute syndrome with immunosuppressive drugs. Prednisone (130); antilymphocyte serum (131); interferon (132); levamisole (133); cyclosporin A (134, 135); and plasmapheresis, azathioprene, and antilymphocyte serum (136) have all been tried. Only cyclosporin appears to have produced worthwhile results to date. Stiller et al (134) recently reported over 60 cases of insulin-dependent diabetes treated at the time of onset with cyclosporin. During treatment, 58% of the patients were successfully withdrawn from insulin therapy for up to a year. The effectiveness of cyclosporin immunosuppression clearly lends further support to the hypothesis that an autoimmune process leads to IDDM. At this writing, the clinical course following withdrawal of cyclosporin therapy is unknown.

In terms of clinical utility, however, the use of any immunosuppressive agent carries with it many risks (137). The most serious is the likelihood of diffuse alteration of the immune system rendering the patient susceptible to infection or neoplasia. Although the data suggest that there is short-term benefit, immunosuppressive agents may have to be given throughout a patient's life in order to prevent recurrence of diabetes. With all the dangers inherent in immunosuppression, the cure could lead to complications as grave as those of the disease.

ANIMAL MODELS OF INSULIN-DEPENDENT DIABETES MELLITUS

With the rapid advances being made in the study of human IDDM and the recent tentative efforts at therapy, it may be somewhat surprising that diabetes research still relies heavily on the study of animal models. Nonetheless, it must be acknowledged that the inheritance, etiology, physiology, therapy, and prevention of human IDDM are still unsolved problems. The complexity and number of these unanswered questions are such that the use of animal models continues to provide valuable insight.

The principal advantages of animal models include the availability of biopsy and autopsy material for study, as well as the ability to record and manipulate modes of inheritance. Animal models also provide the means to surmount the ethical impediments to the study of human IDDM. Provocation of the disease is obviously not permissible in humans, but constitutes a fruitful line of animal research. As a corollary, new therapeutics to prevent or reverse the disease can first be tested in animals, as was the case with cyclosporin.

The advantages of animal models are, of course, balanced by limitations. No animal model of diabetes corresponds perfectly to human diabetes. The clinical course of diabetes in these animals can be variable, unpredictable, and sometimes difficult to relate to human physiology. Choice of an appropriate model is crucial. Although spontaneous hyperglycemic syndromes have long been recognized in a wide variety of species (138), only a very few exhibit convincing evidence of an autoimmune pathogenesis. Foremost among these are the mouse treated with multiple doses of streptozotocin, the nonobese diabetic (NOD) mouse, the mouse infected with encephalomyocarditis (EMC) virus, and the BB rat.

STREPTOZOTOCIN-INDUCED INSULITIS

If a single small dose of the beta-cytotoxic drug streptozotocin (SZ) is given to mice, there is little effect. If, however, SZ is given in multiple small doses, it produces pancreatic insulitis with progression to nearly complete beta-cell destruction and severe diabetes (139). The development of hyperglycemia and insulitis is associated with increased numbers of Type C viruses in the pancreatic beta cells. Differences in strain susceptibility have been related to the major histocompatibility complex (H-2) (140), though other studies have failed to provide confirmation (141). Males have been shown to be more susceptible than females.

An immune component to the pathogenesis is suggested by the observation that antimouse lymphocyte serum protects mice from SZ insulitis (142). Total body irradiation also inhibits the disease process (143),

and athymic mice are not susceptible to multidose SZ, while genetically similar mice with an intact thymus response normally (144). Reconstitution of nude mice with thymus transplants renders them susceptible to the syndrome. Additional immunosuppressive agents such as inosiplex decrease susceptibility in this model (145).

Nonetheless, other studies suggest that the insulitis may be the consequence and not the cause of beta-cell destruction (146), and streptozotocin-induced insulitis is at best an equivocal model of autoimmune diabetes.

THE NONOBESE DIABETIC (NOD) MOUSE

This model of diabetes was derived from the CTS mouse in Japan. The animals are lean and ketosis prone. They develop insulitis at approximately five weeks of age, and by seven months of age 80% of female and 20% of male mice are affected. NOD mice have reduced numbers of T lymphocytes, natural killer cells, and cytotoxic lymphocytes (147). Islet-cell antibodies have been found as early as two weeks of age. Inflamed islets contain a high proportion of cytotoxic/suppressor T lymphocytes and helper/inducer lymphocytes (148). Nicotinamide injections reportedly prevent the diabetes (149), but the mechanism of protection is not clear. The relevance of this model in the study of human IDDM remains to be established.

ENCEPHALOMYOCARDITIS (EMC) VIRUS INFECTION DIABETES

Injection of the M variant of EMC virus into certain strains of adult male mice causes, with an efficiency of about 40% (150), an insulin-dependent diabetes-like syndrome. Pathologically, pancreatic insulitis is observed, suggesting a cell-mediated immune pathogenesis. This hypothesis is supported by the finding that radiation or the administration of ALS will ameliorate the syndrome (151, 152). Other studies have demonstrated that susceptibility to this infection may be mediated by the production of interferon. This model is particularly valuable as a tool for investigating the possible role of viruses in the initiation of IDDM.

THE BB RAT

To date, the best analog of human IDDM is provided by the BB rat (153). It was discovered in 1974 in a commercial colony of Wistar-derived rats maintained at the Bio-Breeding Laboratories of Ottawa, Canada. The original BB colony was established by crossbreeding the normal parents of diabetic animals. Initially, 10% of the offspring of these matings became

diabetic, but with selective breeding the frequency of diabetes has been substantially increased. All BB rats are descended from the original Ottowa litters, but rats in different colonies vary in the frequency and severity of diabetes.

Natural History

Onset of diabetes in the BB rat is abrupt. Most diabetic animals succumb from ketoacidosis within one week of onset unless insulin is given. A small number of animals manifest only hyperglycemia and do not require exogenous insulin (154). Variable but usually small numbers of animals become glucose intolerant and do not progress to overt diabetes. The age at which diabetes develops is relatively consistent, generally between 60 and 120 days of age in most colonies. Among animals bred at the University of Massachusetts in Worcester (designated BB/W rats), fewer than 0.5% become diabetic before 60 days of age. Approximately 40–50% of BB/W rats become diabetic between 60 and 120 days of age; about 15–20% become diabetic after 120 days of age. Diabetes occurs equally in both sexes, and obesity is absent.

Prior to the onset of diabetes, some animals evidence abnormal insulin secretion and some are glucose intolerant. In other animals no prodromal abnormalities are observed. Once the animals are persistently glycosuric they become permanently diabetic. Insulin-dependent rats that spontaneously enter remission are very rare (154).

Physiologic and Biochemical Features

Plasma glucose levels in diabetic BB rats are greater than 250 mg/dl and are often greater than 500 mg/dl. Ketone body and circulating free fatty acid concentrations are also elevated. Insulin levels are markedly depressed (155). Hyperaminoacidemia and a generalized catabolic state are suggested by elevated 3-methylhistidine and branched chain amino acid concentrations and by increased ammoniagenesis. In the prediabetic state, growth hormone dynamics are normal.

Genetics

Once the animals became available, programs to inbreed by brother/sister matings were begun. At the University of Massachusetts there are now (1984) five family lines of BB/W rats in their eighteenth to twentieth generation of inbreeding. In addition, there are three lines of BB-derived rats that have been largely free of diabetes for more than 13 generations. To date there have been 25 diabetics among >1500 rats tested. These lines are designated the diabetes-resistant lines or W-lines and were derived from diabetes-prone forebears.

At the University of Massachusetts, the age at onset of diabetes has not

changed significantly with inbreeding, nor has the severity of the diabetes. Mating of diabetics with diabetics increased the incidence to 45–60%, but with further inbreeding, there has been no change. The mechanism of inheritance is thought to be a recessive gene or gene cluster with 50% penetrance (156). The data to support this contention come from diallele tests in which diabetic and normal offspring of two diabetic parents have the same genotype (156).

Crossing the BB rat with most non-BB inbred or outbred rats has failed to yield F_1 diabetics. The only exception is a single cross between the Buffalo rat and the BB. However, in F_2 generations, the frequency of diabetes is 2–5% (157, 158). At least two genes are necessary for the development of diabetes (157). One is associated with the major histocompatibility complex (RT1) and the other is related to T cell lymphopenia (see below). A third component, pancreatic lymphocytic infiltration, has also been implicated as a separate factor required for the full expression of the diabetic state (158). A recent study has employed restriction endonucleases to determine RT1 class II–gene polymorphisms in the BB rat (159). Using a I-A alpha mouse MHC gene probe, four polymorphic chromosome types were found in diabetes-resistant BB rats from the Ottawa colony. All diabetes-prone and -resistant BB rats from the Worcester colony were homozygous (II-A/II-A). The possibility that a class I antigen may be more closely associated with diabetes has also been suggested (160).

Morphological Findings: Insulitis

The pancreatic islets of all acutely diabetic BB rats are infiltrated by mononuclear cells. Many normoglycemic BB rats also exhibit insulitis of variable intensity. This is true of both younger, presumably prediabetic rats and of older nondiabetics. Periductular and/or acinar aggregates of lymphocytes and macrophages are also present in diabetic and nondiabetic rats and are thought to precede the development of insulitis. The development of severe insulitis precedes and coincides with the appearance of acute diabetes (Table 2). Biopsies of pancreata from animals 2–9 days prior to the acute diabetic state show significant insulitis (161), as early as 45 days of age.

Islets from rats sacrificed at the time of onset of diabetes contain virtually no beta cells. The number of A, D, and PP cells is also decreased. Extraction of islet hormones from diabetic rats confirms the absence of insulin and the decrease in other islet hormones (162). Insulitis is generally absent in long-term, insulin-treated diabetic rats.

In animals with insulitis, B lymphocytes are essentially absent from the islets. The majority of infiltrating inflammatory cells react with OX4

Table 2 Prospective studies demonstrating morphological and humoral changes in the BB rat

	Age (days)						
	45	50	60	75	85	105	120
Insulitis[a]	0		0	50%	43%	43%	67%
Thyroiditis[a]	0		0	10%	9%	0	24%
Anti-thyroid colloid antibodies[a]	0		55%	70%	57%	79%	76%
Anti-smooth muscle antibodies[a]	0		35%	5%	22%	43%	95%
Anti-islet cell surface antibodies (protein A)[b]		38%					
Anti-lymphocyte antibodies[b]		77%					

[a] Appel, M. C. (personal communication). Twenty or more animals were studied prospectively.
[b] Dyrberg, T. et al (185).

monoclonal antibody, indicating that they are Ia^+ cells (163). This implies that they are macrophages and/or activated T cells. Other cells present, in decreasing numbers, include helper-inducer cells and cytotoxic-suppressor cells.

Environmental Factors

The BB rat has afforded the opportunity to study the early phases of induction of diabetes. To test the possibility of an infectious etiology, these animals have been raised in a sterile environment (164). Under gnotobiotic conditions it was possible to exclude viral and bacterial pathogens on the basis of negative cultures, stained smears, and serologies. Despite the sterility of the environment, the expected percentage of rats became diabetic. This experiment implies that infection is not a prerequisite for the development of the IDDM-like syndrome and suggests that genetically mediated factors are more important. This study, however, does not exclude the possibility of either a vertically transmitted virus or a slow virus in the pathogenesis.

Other hormonal and environmental factors have also been evaluated (165). Hypophysectomy, castration, vagotomy, and 3-O-methyl-D-glucose (an agent known to protect against beta cytotoxins) did not protect against diabetes. Similarly, nicotinamide and stress produced by ultrasonic noise did not alter the incidence of diabetes. Diets high in carbohydrate, protein, or fat did not influence the incidence of diabetes in one report (165), but a more recent study used a semi-synthetic diet in which natural proteins were replaced by L-amino acids. This diet, if begun at weaning, reduced the incidence of diabetes from 50% to 15% (166). The mean age at onset in this

study was also delayed. It is unclear if the diet itself or impaired growth afforded this protection. In hypophysectomized animals with growth retardation the mean age at onset was 130 days (165). Additional experiments will be necessary to determine the role of dietary factors in the pathogenesis of diabetes in the BB rat.

Immunologic Abnormalities

Lymphopenia has been a nearly universal finding in all colonies of BB rats (167, 168). It occurs postnatally (169). As mentioned above, a number of studies have suggested that lymphopenia is essential for the development of diabetes in this animal. Using monoclonal antibodies, a number of laboratories have demonstrated that there is profound T lymphopenia in both the peripheral blood and the lymphoid tissues of BB rats (169). Several investigators have reported that helper T cells are the most significantly depleted of the T lymphocytes (168, 172), while others have reported that the predominant deficiency is in cytotoxic-suppressor (OX8) cells (163, 173). B lymphocytes, as determined by surface Ig, are reported to be decreased or normal when compared to control animals. There may, however, be a relative increase in the percentage of B lymphocytes (170, 171).

Functionally, the response of BB rat lymphocytes to con A and other mitogens is impaired (170, 171, 174). Mixed lymphocyte reactions are also impaired (171, 175). Interleukin-2 production was reported in one early study to be normal, but administration of IL-2 did not enhance the depressed con A stimulation of T lymphocytes (171). More recent studies contradict these observations, suggesting an inhibition of IL-2 production caused by macrophages (176). This defect in IL-2 production has been confirmed by another laboratory which demonstrated normalization of the blunted MLR and con A responses of BB lymphocytes following the addition of IL-2 (177). Another T-cell functional abnormality observed in these animals is delayed skin allograft rejection (171).

Examination of peripheral lymphoid tissues, including spleen and lymph nodes, has demonstrated profound depletion of T lymphocytes in these organs. The histology of the thymus gland is reportedly normal (171), but a more recent study using flow cytometry demonstrated an increased number of large thymocytes (179). The importance of this preliminary observation is unclear.

Concomitant with the severe lymphopenia, BB rats are susceptible to environmental pathogens (180, 181) and malignant lymphomas. One study has suggested that B-cell lymphoproliferation is more common in chronically diabetic rats than in nondiabetics. However, other studies report lymphomas in approximately 4% of both diabetic and nondiabetic animals

(182). Autopsy studies of diabetic BB rats revealed 30% of the animals to have significant pneumonia (181, 182). Our colony was once decimated by a widespread epidemic of mycoplasma.

Data Implicating an Autoimmune Pathogenesis

The observation of insulitis was only the first of many lines of evidence to support the hypothesis of an autoimmune pathogenesis of diabetes in the BB rat. Other important supportive data have come from studies of humoral and cell-mediated immunity.

Humoral Immunity

Classic islet-cell cytoplasmic antibodies have not been found in the BB rat (180). Islet cell–surface antibodies have been detected by indirect immunofluorescence using pancreatic islet-cell suspensions from cultured rat islets (183). Complement fixing–antibodies (CFA) against surface antigens on dispersed Wistar Furth islet cells have also been observed in the serums of diabetics (184). CFA usually appear up to two weeks before the appearance of frank diabetes. Using a radiolabeled protein A binding technique, islet-cell antibodies are detectable at 40 days of age, and 75% of the animals had them by 74 days of age (185). Another antibody that immunoprecipitates a 64 kd rat islet-cell protein similar to that in humans (186) has been described; it also precedes the onset of diabetes (187).

Antilymphocyte antibodies are prevalent in diabetic animals (185) as are autoantibodies to smooth muscle, thyroid colloid, and gastric parietal cells (180, 188). The age at appearance of these antibodies is summarized in Table 2. Thyroiditis is also common in BB rats, but no thyroid functional abnormalities have been found (189). Antibodies to the pituitary, adrenals, testes, and ovaries are absent, and no inflammation in these tissues has been observed. Data on the prevalence of autoantibodies and thyroiditis are given in Table 2.

Cell-Mediated Immunity

To help establish that a disease is a cell-mediated immune process it is desirable to demonstrate that it can be transferred to unaffected hosts using the presumed effector cell. It is also important to show that immunosuppressive agents or immune modulation can protect against the disease.

PASSIVE TRANSFER Peripheral and splenic lymphocytes from newly diagnosed diabetic BB rats were first reported to transfer insulitis passively when given to athymic mice (190). Repeated injections of the cells increased the frequency and the intensity of the insulitis. However, no hyperglycemia

could be detected in the recipients, even when stressed with a glucose tolerance test. A comparable study using a different strain of athymic mice failed to confirm these findings (191).

Adoptive transfer of both insulitis and diabetes was accomplished by a technique first devised for the study of experimental allergic encephalomyelitis (192). Spleen cells from acutely diabetic BB/W rats were cultured in the presence of con A for three days. The transfer was effected by administering the cells to 30-day-old, diabetes-prone rats. As noted above, fewer than 0.5% of BB/W rats develop diabetes before 60 days of age. Most rats given activated lymphocytes, however, developed diabetes within 2–3 weeks, or by 50 days of age.

Additional experiments have studied the role of class II antigens in this process. The BB rat and the W-line rat share the same $RT1^u$ haplotype as the Wistar Furth rat. It has now been demonstrated that diabetes can also be transferred to Wistar Furth rats and to the diabetes resistant W-subline of the BB rat and to F_1 (BB × non $RT1^u$) hybrids if the recipients are first pretreated with cyclophosphamide or another immunosuppressive method (193, 194). Thus, by altering the immune system of the recipient, diabetes can be transferred into non BB animals which are homozygous or heterozygous for $RT1^u$.

In another experiment, rats with various RT1 haplotypes and a congenic line of rats were lethally irradiated and then reconstituted with bone marrow from diabetes-prone BB rats. These animals then received con A–activated splenocytes from acutely diabetic BB rats. Only animals with the $RT1^u$ haplotype became diabetic (196), though some non $RT1^u$ animals were subsequently found to have insulitis. These observations have been interpreted to suggest that adoptive transfer of diabetes but not insulitis may be MHC restricted.

It has also been demonstrated recently that the frequent administration of conditioned media obtained from the incubation of splenocytes with con A can induce diabetes before 60 days of age in both diabetes-prone BB/W rats and diabetes-resistant W-line BB rats (197). Injections of unconditioned media containing con A were ineffective. These data suggest that a soluble substance can induce diabetes.

IMMUNOSUPPRESSIVE AGENTS Various immunosuppressive regimens are effective in preventing and ameliorating BB rat diabetes. In one experiment, acutely diabetic rats and nondiabetic littermates were given either rabbit antirat lymphocyte serum (ALS), 500 rads of total body irradiation, or no treatment (195). Normalization of blood glucose occurred in 36% of ALS-treated animals and 13% of irradiated animals, but in none of the untreated controls. In addition, nondiabetic littermates given treatment failed to

develop diabetes in expected numbers, suggesting that susceptible animals had been protected from the disease. The combination of ALS with long-acting glucocorticoids also prevents diabetes, as does the combination of cyclosporin with other immunosuppressive agents (198). Total lymphoid irradiation is another effective method of prevention in the BB rat (199).

Cyclosporin alone alters the development of the diabetic state. Weanling diabetes-prone rats were given cyclosporin orally to maintain serum levels between 100 and 300 μg/ml (200). The animals were followed for 120 days and no treated animals became diabetic, as contrasted with 75% of controls. Once therapy was discontinued 38% of females and 13% of males spontaneously became diabetic during the following 49 days. In another study, prophylactic cyclosporin given at 10-day intervals prior to 70 days of age reduced the frequency and delayed the onset of diabetes. These data suggest that treatment during a relatively narrow period may alter the autoimmune process (201). Cyclosporin administered at the onset of diabetes did not cure existing diabetes.

Cyclosporin is believed to act by inhibiting T-lymphocyte proliferation in response to T cell–dependent antigens. It affects principally the helper/inducer T lymphocytes while sparing suppressor lymphocytes. The suppressive effect may predominate, attenuating the effector-cell population and sparing the target cell, in this case the beta cell.

NEONATAL THYMECTOMY Another approach that prevents diabetes in the BB rat is neonatal thymectomy. Animals thymectomized within 24 hr of birth had a reduced frequency of diabetes in later life (202). The data again suggest the T lymphocytes are involved in the pathogenesis of the diabetic syndrome.

IMMUNE ENHANCEMENT Bone marrow from Wistar Furth rats transplanted to neonatal BB rats decreased the frequency of diabetes (203). This procedure also corrected the lymphopenia and normalized the mitogenic response of the lymphocytes (204). It is possible that a chimeric cell population was established, and that a subpopulation of suppressor T lymphocytes subsequently protected against the diabetic syndrome.

Transfusions of whole blood from the diabetes-resistant W line of BB rats also prevent diabetes in susceptible BB rats. The initial observation was that 11 weekly transfusions of whole blood prevent spontaneous diabetes in the BB rat if treatment was begun at about 30 days of age (174). Subsequent studies have shown that as few as five transfusions are needed. Experiments were conducted to determine which blood component is protective (206). The white cell component not only protected against diabetes, but also restored the lymphocyte count and responsiveness to con A. Erythrocytes and plasma were ineffective. Additional experiments showed that blood

from the low-incidence W line treated with a cytotoxic anti-T-lymphocyte antibody plus complement removed the protective lymphocyte component. Finally, the specificity of protection was demonstrated in experiments showing that spleen cells from the W line were protective while those from nondiabetic but diabetes-prone rats were not. These results suggest that a T lymphocyte can confer protection from diabetes.

It seems likely that there exists a spectrum of immunodeficiency in the BB rat ranging from the acutely diabetic animal with a preponderance of effector cells to the W-line genetic mutation in which protective cells predominate.

ISLET TRANSPLANTATION Islets of Langerhans from diabetes-resistant BB rats transplanted to a spontaneously diabetic BB rat are rapidly destroyed, suggesting that the transplanted islets are vulnerable to the original disease process (207). To determine if destruction of transplanted beta cells is due to an abnormal antigen, islets from the diabetes-resistant W line were isolated and treated using a standard technique to prevent allograft rejection. The islets were implanted into the kidney capsule of rats with diabetes of brief (1–2 days), short-term (21 days), or long-term (90 day) duration (207). The transplanted islets evidenced lymphocytic insulitis most severely in the acutely diabetic host, least in the short-term diabetic, and to an intermediate degree in the long-term diabetic. This suggests that an abnormal beta-cell antigen is not essential for initiating insulitis and that the intensity of immune attack on islet transplants decreases with duration of diabetes. To determine if the attack on the transplanted islets is MHC restricted, islets from a number of non-RT1^u inbred animals were treated to prevent allograft rejection and transplanted into diabetes-prone recipients (208). Insulitis in the graft was observed in rats of all haplotypes, but only when the recipient was either diabetic or had evidence of endogenous pancreatic insulitis. These results have been interpreted to suggest that insulitis in the BB/W rat is not MHC restricted.

FUTURE APPROACHES TO ANIMAL AND HUMAN DIABETES

One may reasonably ask if the data obtained in rat models apply to humans. The answer is a qualified yes. For example, human clinical trials using cyclosporin were begun after successful use of the drug in the BB rat. But it is important to remember that human IDDM is still an incompletely understood disease. This is evidenced by the lack of congruity among much of the immunologic data reviewed here. Biases in patient selection and the limitations of current technology also contribute to our uncertainty about

the exact nature of the immune defects in IDDM. Truly specific therapy for either the rat or the human will require the identification and isolation of the specific effector cell. To date this has not been achieved in either species. If this were to be achieved, however, production of specific monoclonal antibodies to deplete these effector cells might well provide effective specific treatment. At the present time broadly immunosuppressive agents may be effective, but they pose the risk of major side effects.

If it were to be demonstrated conclusively that there are immunologic deficits in humans with IDDM, then it would be preferable to treat IDDM with immunoreconstitution (e.g. lymphocyte transfusion) rather than immunosuppression. It can always be argued, of course, that lymphocytes with different haplotypes or even minor histocompatibilities would be rejected, but the animal data suggest that this procedure might be feasible in humans. In the BB rat model immune-modulation by lymphocyte transfusion has been demonstrated and the data suggest that a lymphocyte chimera was produced. The low-incidence, W-line donors were originally derived from diabetes-prone rats, and the histocompatibility of donors and recipients was probably very great. Lymphocytes from the W-line rat transfused into the immunodeficient diabetes-prone BB/W rat are unlikely to have been rejected. If transfused lymphocytes from the resistant donor rats had been rejected by the diabetes-susceptible recipients, they would probably not have conferred protection.

In approaching human IDDM, the most logical alternative to the allogenic transfusion achievable in the rat would be the use of identical twins as the donor-recipient pair. Since these individuals are syngeneic, they would be analogous to the diabetes-prone and W-line BB rats used in transfusion studies. They could be capable of establishing an inter-twin chimerism. Since insulin-dependent diabetes in identical twins has a concordance rate of only 30–50%, it is possible that a twin discordant for diabetes may have a lymphocyte population that could protect against the autoimmune process in a diabetic monozygotic sibling. If so, one might speculate that transfer of lymphocytes from one twin to another could prevent or ameliorate insulin-dependent diabetes. Studies to test this hypothesis are currently under way.

There are many other directions for future research. It is possible that the lymphocytes in diabetic children may be defective in producing lymphokines. There are already data to suggest that this is true. An alternative to immunosuppressive therapy could involve the administration of a specific lymphokine.

Current data suggest also that beta-cell numbers are markedly reduced at the onset of clinical diabetes. The development of procedures to identify high-risk individuals accurately is an obvious necessity. This may ulti-

mately be achieved through the identification of the gene or genes responsible for susceptibility.

There are many other unanswered questions. What triggers the immune-response gene? Why is autoimmune diabetes so prevalent in the young? (In the life cycle of a rat the age of onset of diabetes is comparable to that of the human; both occur in adolescence.) Hopefully, better technology and more work will provide answers.

SUMMARY AND CONCLUSION

We have attempted to illustrate the major trends in research in human IDDM and in the animal models of this disease. We have stressed the utility and importance of the animal models. Their contribution to understanding the human disease is great but the limitations inherent in their use should also be evident. Extrapolation from rodent to human is always treacherous. Nonetheless, the animals still offer great promise in unraveling the pathogenesis of diabetes. The excellent results obtained in BB rats have already led to the use of cyclosporin in human trials. It is clear that we have learned a great deal from diabetic animals and that they have a good deal yet to teach us. Hopefully, an enhanced understanding of the pathogenesis of human insulin-dependent diabetes will follow and lead to preventive and curative therapies that are both safe and effective.

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CONTENTS

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HUMAN T-CELL LEUKEMIA VIRUSES (HTLV): A Unique Family of Pathogenic Retroviruses

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INTRODUCTION

The term human T-cell leukemia virus (HTLV) denotes a unique family of T-cell lymphotropic, pathogenic retroviruses. The discovery of HTLV-I, a virus belonging to this family, in 1978 by workers in the Laboratory of Tumor Cell Biology at the National Cancer Institute led to the first proven link between retroviruses and cancer in human beings (1–3). This discovery also led to concepts and technologies that made it possible later to define another member of the HTLV family, HTLV-III, as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (4–7). In this chapter, we review how clinical perspectives have influenced the analysis of the host/retrovirus interactions at a research level, and by the same token how research perspectives have influenced clinical knowledge of certain disorders characterized by an uncontrolled proliferation or ablation of T cells.

Clinical Perspectives

In 1977, Uchiyama and his colleagues (8) described a rapidly fatal T-cell lymphoproliferative syndrome affecting adults born in the southwestern

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portion of Japan. The authors referred to this syndrome as adult T-cell leukemia (ATL). The syndrome is characterized by the presence of circulating neoplastic T cells with the membrane markers of helper/inducer ($OKT4^+$) T cells, although in functional assays such cells tend to suppress mitogen-driven immunoglobulin production by cocultured normal B cells. Other common findings include lymphadenopathy, hepatosplenomegaly, cutaneous infiltration with neoplastic T cells, hypercalcemia (with or without detectable lytic bone lesions), and a predisposition to infections, with opportunistic infections occurring even before the administration of systemic chemotherapy (9). Mediastinal tumors are not a feature of this syndrome. The remarkable geographic clustering of ATL cases and the special clinical features induced these Japanese investigators to conclude that this disease represented a new category of malignancy which differed from the Sézary syndrome and other T-cell neoplasms. However, a specific cause could not be ascribed for this newly recognized endemic form of cancer.

At approximately the same time, the Laboratory of Tumor Cell Biology at the National Cancer Institute (1–3, 10–13) was studying American patients who had been diagnosed by referring physicians as having mycosis fungoides/Sézary syndrome. Some of these patients, in retrospect, had clinical and laboratory features of the ATL seen in Japan. These American patients yielded the first human, type–C RNA, tumor virus (retrovirus), now designated HTLV-I (3).

The occasional identification of HTLV-I in patients seen at the National Cancer Institute, coupled with the unique clinical and epidemiologic features of ATL in Japan, stimulated Gallo and his colleagues to hypothesize that HTLV-I was a transmissible agent responsible for this endemic T-cell neoplasm (3). When specimens from southwestern Japan were analyzed, essentially every case of ATL had evidence of HTLV-I (but the incidence of infection was quite low in appropriate control groups). Hinuma and his coworkers reported comparable findings shortly thereafter. Subsequently, similar results were obtained in patients from other parts of the world (especially the West Indies) where ATL was being recognized as a new entity (3, 14–16). These results provided molecular biologic criteria for diagnosing a neoplastic syndrome previously defined in clinical terms alone.

As progress in understanding the pathogenesis of ATL was being made, approximately three years ago clinicians interested in cancer and immunodeficiency states began to recognize an apparently unrelated disorder called acquired immunodeficiency syndrome (AIDS) (17–29). The prompt worldwide recognition of this syndrome was brought about by a remarkable collaboration between practicing physicians in several parts of the United States and epidemiologists at the Center for Disease Control

(CDC). The disorder is a transmissible immunosuppressive disease that predisposes the patient to life-threatening infections with opportunistic organisms, Kaposi's sarcoma, and to a lesser extent non-Hodgkin's lymphomas. Characteristically, AIDS is associated with a depletion of T cells, especially the helper/inducer subset bearing the OKT4 surface marker, and in one sense the disease represents the opposite of the $OKT4^+$ T-cell expansion seen in ATL.

The initial outbreak of AIDS in the United States predominantly affected homosexual men and was most severe in cities with large gay populations such as New York and San Francisco. However, AIDS is now a truly international, pandemic disease. To date, 6000 cases have been reported in the USA alone. Following the initial report of AIDS in homosexual men, the CDC formed a task force to study the pandemic systematically. Since no etiologic agent was known, the CDC adopted a surveillance definition based entirely on clinical criteria, which is still in effect. For surveillance and reporting purposes patients are defined as having AIDS if the following conditions apply:

1. At less than 60 years of age, the patient has a reliably diagnosed disease (such as *P. carinii* pneumonia or Kaposi's sarcoma) that suggests an underlying cellular immune defect.

2. The disease occurs in the absence of a cellular immune deficiency that can be ascribed to another factor, e.g. the use of immunosuppressive drugs or a prior lymphoreticular malignancy.

Most cases of AIDS have occurred in patients belonging to one of four categories that are referred to as risk groups for epidemiologic purposes: (*a*) sexually active homosexual or bisexual men (roughly 75% of cases), (*b*) present or past abusers of intravenous drugs (about 17%), (*c*) Haitian entrants into the USA (about 5%), and (*d*) individuals with hemophilia (<1%). AIDS has developed in patients receiving even small numbers of blood transfusions, and cases of apparent transplacental transmission have been reported (25, 26). Moreover, in certain parts of the world, such as Central Africa, the disease may be occurring in individuals who do not belong to one of the known AIDS risk groups (28).

Several points are worth stressing in connection with African AIDS. There is strong evidence that AIDS or illnesses related to AIDS exist in several Central African countries, including Zambia, Zaire, and Rwanda (30–32). Indeed, in one published report, the AIDS rate in Kigali (the capital of Rwanda) is estimated to be 80/100,000 (32); if confirmed by further studies, this would represent an astonishing incidence. While it is impossible to date precisely the onset of AIDS in Africa, most known cases have occurred since 1981, which is consistent with the simultaneous emergence of AIDS cases in the USA and Africa. There is essentially an equal proportion of male and female African AIDS patients, and it would

appear that *heterosexual* contact is the most frequent mode of transmission of the disease in Central Africa. Moreover, both male-to-female and female-to-male heterosexual transmission can occur. Many of the patients in Central Africa are comparatively well-to-do professionals, who worked in urban centers. An urban setting, a comparatively high socioeconomic status, and heterosexual promiscuity are risk factors for African AIDS. In light of these observations and others we will discuss later, one can infer that heterosexual transmission will become an increasingly important factor in the epidemiology of AIDS in other parts of the world, including the United States.

While a great deal of clinical knowledge accumulated on AIDS, a major frustration for physicians and scientists who dealt with this pandemic was the inability to define an etiologic agent. This difficulty restricted progress at virtually every level. However, in the spring of 1984 several converging lines of investigation linked a newly discovered member of the HTLV family (HTLV-III) to the pathogenesis of AIDS.

In the remainder of this chapter, we discuss some of the recent research developments in understanding members of the HLTV family and their relationship to specific disease processes. We will give special emphasis to the role of HTLV-III in AIDS because this disease may become among the most important pandemics of our time.

Research Perspectives

The current discoveries can perhaps best be understood in the larger context of retrovirus research (1–3, 10–16, 33, 34). A retrovirus encodes its genetic information in RNA and uses a unique viral enzyme called reverse transcriptase to copy its genome into DNA, which is then integrated into the host cell genome as a provirus. One can observe amino acid–sequence similarities when the reverse transcriptases of both animal and human retroviruses are compared (35). One can also see interesting similarities between certain reverse transcriptases and a common pathogenic DNA virus, hepatitis B virus (HBV), probably involving a region that contains the genetic information for a DNA polymerase (35, 36).

Usually retroviruses contain three genes, including a gene for the viral internal core proteins called *gag*, the polymerase or reverse transcriptase gene called *pol*, and the gene for the surface envelope protein called *env* (3). The transcription initiation signals for a retrovirus lie within the long terminal repeat (LTR) sequences that flank the integrated provirus. Certain viral genes are sometimes replaced by a cell-derived DNA sequence known as an *onc* gene, which enables some animal retroviruses to acutely transform appropriate target cells. [However, members of the HTLV family of human retroviruses as well as the vast majority of naturally occurring pathogenic retroviruses of animals do not carry known *onc* genes (3).]

Retroviruses have been unequivocally linked to certain cancers and immunodeficiency states in animals, but evidence for human counterparts of the animal retroviruses was slow to develop. Indeed, many individuals questioned the very existence of human retroviruses or their relevance to human disease altogether. Beginning in 1970, after the discovery of reverse transcriptase (37, 38), molecular and biological approaches to detect low-level expression of retroviruses applicable to the study of human tissues (39), as well as techniques for the routine long-term culture of various hematopoietic cells, were developed in the Laboratory of Tumor Cell Biology at the National Cancer Institute. Both technologies proved useful in the first isolation and identification of human retroviruses in the United States, and soon thereafter in several parts of the world.

The long-term growth of mature human T cells was made possible in 1976, when a protein called T-cell growth factor (TCGF) was identified (40). This protein, also later called interleukin 2 (IL-2), has been purified, its gene cloned, and its membrane receptor characterized (41–43). It was soon apparent that most primary normal T cells do not have receptors for TCGF until stimulated by antigen or lectin (9). However, T cells from some adult T-cell leukemias contain abundant receptors. By using TCGF it became possible routinely to establish T-cell lines from normal individuals and from patients with various diseases including certain T-cell malignancies. Cultured cells derived from American patients with TCGF receptor–positive adult T-cell leukemias were the source of the first isolates of human retroviruses in 1978 and 1980. Large-scale virus production in the laboratory was possible at the outset. These isolates were generically called human T-cell leukemia virus (HTLV) when the work was published in 1980 (1) and 1981 (2), after immunologic, biochemical, and genetic analyses had proven that the agent was not an animal virus contaminant (10–13), and that it could be detected repeatedly in the same patient. In culture, this unique virus could infect and transform human T cells (9, 15, 44). T cells infected by the virus were noted to produce a variety of lymphokines constitutively (45), and it is likely that the production of a special lymphokine called osteoclast-activating factor (OAF) is responsible for the hypercalcemia seen in patients with adult T-cell leukemia (46). In certain settings, infected cells acquired new functional properties, including the capacity to respond to soluble antigen in the absence of accessory cells (47). In other settings, however, infection led to a cytopathic effect in a given target cell (48). In some individuals, infection can lead to an HTLV-specific cellular immune response, restricted by major histocompatibility antigens (49, 50). One topic for future clinical research is to explore the role of adoptive cellular therapy with autologous immune T cells, alone or in combination with other modalities of therapy.

Two immunologic features of the virus are quite interesting, but not as

yet completely explained. First, the viral p19 *gag* protein appears to cross-react with a determinant found within the neuroendocrine portion of normal human thymic epithelium (51) and also in syncytiotrophoblastic cells of first trimester human placentas (52). Second, there is a preferential association of purified (extracellular) virions with the receptor of TCGF (53).

Soon after the isolation of the first human retrovirus, HTLV was found to be endemic in southern Japan, Africa, and certain parts of the Western Hemisphere, including the southeastern portion of the United States, and especially the West Indies; and it was etiologically linked to the origin of adult T-cell leukemia (ATL) (9).

Later it became apparent that HTLV should be viewed as a *family* of related acquired T-lymphotropic retroviruses, not as one virus. The prototypical member of the family (first isolated from American, Caribbean, and Japanese patients with ATL) is referred to as HTLV-I. The second member of the family is designated HTLV-II (34).

The genes of HTLV-I and HTLV-II have been cloned and analyzed in detail (33, 54, 55), and the sequence of the corresponding HTLV-I and -II envelope genes (which are the major antigens recognized by infected patients) are similar (56). Several novel features were found which might make the HTLV family taken together one of the most interesting collections of all known retroviruses, perhaps transcending the significance in the pathogenesis of any one disease. We shall try to touch upon some of the special molecular and biological features of the HTLV family.

These viruses do not possess known *onc* genes, and yet (in the case of HTLV-I and HTLV-II) are acutely transforming in vitro. The most frequently identified member of the HTLV family is HTLV-I (we are aware of more than 100 isolates to date). Moreover, HTLV-I is the only member of this family identified in Japan to date, and the virus in Japan is identical to the HTLV-I found in the USA and the Caribbean, implying either a relatively recent separation or unusual biologic imperatives that prevent divergence.

Several features related to the genetic organization of the HTLV-I provirus and its pattern of insertion into the host genome are worth noting. There is only one provirus integrated into the DNA of a given host cell (9, 57, 58). Although HTLV-I can integrate into numerous different chromosomal loci, analysis of HTLV-bearing leukemias and lymphomas has shown that the viral integration is monoclonal, i.e. each tumor cell in a single patient has the viral sequence integrated in the same place. We wish to stress one point: Since HTLV-I can integrate in many different chromosomal loci (59, 60), these findings taken together demonstrate that HTLV-I infects the individual *before* the neoplastic transformation of the first T cell. In other words, the process does not reflect a late passenger-virus

effect, and this supports a direct role for HTLV-I in at least one step in the process of leukemogenesis.

The knowledge that HTLV-I has no common pattern of chromosomal integration set the stage for workers in the Laboratory of Tumor Cell Biology and in other laboratories to implicate a process of *trans*-acting transcriptional activation in the leukemogenesis induced by this virus. In this regard, it is necessary to discuss a special segment of the virus called the pX region. To date, all viruses of the HTLV family have a unique pX sequence at the 3′ of the viral genome. In the analysis of one prototype of HTLV-I, the pX region had open-reading frames with the genetic information for four polypeptides (33). However, more recent work with different variants of HTLV-I and comparisons of HTLV-I with HTLV-II indicate that there is a 3′, 1011-nucleotide long region which is highly conserved and has but one open-reading frame [F. Wong-Staal, L. Ratner, G. Shaw, R. C. Gallo, unpublished; (61)]. The pX region contains the genetic information for a protein with a molecular weight of about 40,000, and such a protein likely increases transcription from the viral promoter in the LTR [T. Lee, and M. Essex, unpublished; (61, 62)]. A key working hypothesis in current HTLV research is that such a protein would serve as a regulatory factor that influences transcription from the viral promoter irrespective of the location in the genome. Such a process is denoted by the term *trans*-acting transcriptional activation, and a similar kind of regulatory control mechanism is known to exist for DNA tumor viruses [e.g. adenovirus (63) and SV40 (64)]. A regulatory factor that stimulates transcription from a retroviral promoter would result in an increased rate of viral replication through what is, in effect, a positive feedback loop. Such a factor might also be expected to increase transcription of cellular genes, such as those that participate in mitosis, and thereby be *one* factor in initiating the uncontrolled growth of transformed cells. However, it is very common to observe that freshly harvested tumor cells (known to possess integrated HTLV-I DNA) fail to express detectable viral genes (F. Wong-Staal, G. Franchini, and R. C. Gallo, unpublished). Taken together, these results suggest that the pX gene expression may be necessary in the early steps of transformation, but other factors, not currently defined, may be necessary to maintain the transformed state.

It is worth recapitulating certain very recent data regarding the HTLV family. HTLV-I and HTLV-II have some sequence homology across the *entire* genome. This is especially noteworthy in segments of the envelope region and, as already discussed, in the pX region, as well as in small regions of the gene for reverse transcriptase and viral structural proteins (55). Also, variants of HTLV-I (called HTLV-Ib) have been found in African patients (B. H. Hahn, G. M. Shaw, M. Popovic, A. L. Monico, R. C. Gallo, and F. Wong-Staal, unpublished), and perhaps future research may reveal clinical

manifestations that can be linked to subtle molecular changes in the virus. Moreover, viruses very similar to HTLV-I are present in many Old World monkeys (65–67), but they can be distinguished from the human retroviruses by molecular analyses. The wide distribution of HTLV-I in Africa and its presence in Old World nonhuman primates supports the hypothesis that the HTLV family originated in Africa and that at least one member arrived in the Americas by the slave trade. Moreover, the presence of HTLV-I in Japan and the identity of this virus to the HTLV-I in Africa, the United States, and the Caribbean are consistent with the speculation that HTLV-I arrived in Japan recently, perhaps brought there by the sixteenth century Portuguese mariners who had contact with Africa and Japan in the same period (68).

In contrast to the numerous isolates of HTLV-I from patients with adult T-cell leukemia and the consistent epidemiological and molecular evidence linking the virus to this malignancy, there is only one isolate of HTLV-II in a patient with leukemia (34) and an additional one in an intravenous drug abuser (B. Hahn, M. Popovic, and R. C. Gallo, unpublished). No interesting seroepidemiological results have been obtained to date. Therefore, no conclusions can currently be made regarding HTLV-II as a cause of any human disease.

One can summarize the HTLV viruses by noting that all members of the family (including HTLV-III, the virus that causes AIDS) have at least eight features in common: (*a*) they are exogenous viruses isolated from mature T cells, especially OKT4$^+$ T cells; (*b*) they infect mature T cells in vitro, although other kinds of cells can serve as targets for infection under certain circumstances (69); (*c*) they have a reverse transcriptase with similar biochemical features;[2] (*d*) they possess cross-reacting antigens; (*e*) they have major core proteins of similar size; (*f*) they exhibit some nucleotide sequence homology under nonstringent hybridization conditions; (*g*) they have a pX sequence at the 3′ end of the genome; and (*h*) they can induce the formation of giant multinucleated T cells.

The hypothesis that a retrovirus might cause AIDS was developed in 1982 and was stimulated by a number of considerations, including the knowledge that another retrovirus, feline leukemia virus, causes immunodeficiencies in feral cats (70). Moreover, well-characterized retroviruses in the HTLV family were already known to have tropism for T cells, to exert cytopathic effects on target cells, to alter T-cell functions, to kill immune T cells in certain situations, to exhibit transmissibility by intimate contact or blood products, and to have Africa as a likely origin. These are features one might expect in a putative AIDS-causing virus. Nonetheless, at the beginning of these studies (and even more recently) several other ideas

[2] E.g. similar size, catalytic activity, preference for Mg^{2+} as divalent cation, etc.

attracted inordinate attention including an etiologic role for amyl nitrate, antigen overload, immunosuppressive properties of sperm, the Epstein-Barr virus, cytomegalovirus, and a fungus [e.g. see review by Haverkos and Curran (71)].

The hypothesis that AIDS is caused by one or more immunosuppressive T-cell tropic retroviruses first received direct empirical support in 1983 (72–75). Essex, Lee, and their colleagues discovered the presence of antibodies to cell membrane antigens of HTLV I–infected T cells in serum samples from more than 40% of patients with AIDS (in the most sensitive range of their assay). This antigen is now known to be part of the envelope of HTLV-I. At the same time, three observations reinforced the possibility that a then-uncharacterized member of the HTLV family might be playing a role in the pathogenesis of AIDS: (*a*) Sinoussi-Barré et al at the Pasteur Institute reported a retrovirus termed lymphadenopathy-associated retrovirus (LAV) isolated from a homosexual man, who was felt to have a possible prodrome of AIDS. These workers, in the first paper describing their findings, concluded that the new retrovirus belonged in the HTLV family, but that it was distinct from the other members then defined (75), although this relationship may not have been stressed in subsequent publications (76). (*b*) AIDS was detected in serums containing antibodies to a membrane (HTLV-envelope) protein while lacking antibodies to certain internal core structural proteins of HTLV-I and HTLV-II. (*c*) The isolation of HTLV-I and HTLV-II from AIDS patients was infrequent, even in the presence of antibodies to HTLV-I envelope determinants.

As these studies were intensified by workers at the National Cancer Institute and at the Pasteur Institute, it became clear that cytopathic retroviruses resembling, but not identical to, the known members of the HTLV family could be detected in a large number of patients with AIDS. In fact, such viruses were detected as early as November, 1982, and several additional isolates were made in February, 1983, by workers in the Laboratory of Tumor Cell Biology (S. Salahuddin, P. Markham, M. Popovic, R. C. Gallo). However, these viruses had properties that made reproducible transmission to other cells and continuous production (both, essential elements in the characterization of such viruses) difficult in the extreme. The inability to propagate these elusive retroviruses reliably on a wide scale impeded research within any given laboratory and collaboration among different laboratories [for example, see discussion by R. Weiss (77)], and threatened to forestall almost any immediate clinical or research application. Case by case reporting (76) provided certain clinical and epidemiologic insights, but there was no certainty that each isolate represented the same virus. Each could have been a different, newly discovered human retrovirus present as an opportunistic infection with no bearing on the pathogenesis of AIDS. Viral isolations were therefore not

published by the Laboratory of Tumor Cell Biology until these issues could be resolved.

One hurdle was overcome by Popovic et al when they identified a neoplastic T-cell target population that was susceptible to infection by the new AIDS-related member of the HTLV family (as free virus) but capable of maintaining high rates of growth and virus production despite the cytopathic effect in other T-cell systems (4). This made possible a continuous harvest of large amounts of virus and offered new research opportunities for four reasons. For the first time, the development of both immunological and nucleic-acid probes of a retrovirus linked to the etiology of AIDS was possible. This in turn made possible the precise definition for the first time of the latest addition to HTLV family, HTLV-III, as a *specific* retrovirus—but one that differed from HTLV-I and HTLV-II on morphologic, immunologic, and genetic grounds. Then it was possible to specify that each of 48 new viral isolates obtained from AIDS (or AIDS-risk) patients in the Laboratory of Tumor Cell Biology represented the same agent, and this has now been done for more than 80 isolates. Third, it was now feasible to begin distributing virus to interested researchers on a regular basis. Fourth, and most important from a practical point of view, it provided the opportunity to produce sufficient antigen for nationwide sero-epidemiologic studies, especially blood bank screening.

HTLV-III was found to share many properties with HTLV-I and HTLV-II. As noted earlier, all tend to infect T cells with the $OKT4^+$ (helper/inducer) phenotype, contain similar reverse transcriptases, possess some cross-reactive antigens, have structural proteins of similar size, and induce T-cell fusion. Also, nucleic acid hybridization analysis by Arya et al reveals homologies among all three members of the HTLV family across the entire viral genome, including the pX region (78), albeit the homology of HTLV-III to the other known members of the HTLV family is somewhat distant. It is worth noting that HTLV-III (unlike its relatives in the HTLV family) does not appear to have a transforming capacity for human T cells. Collaborative studies and exchanges between the National Cancer Institute and the Pasteur Institute are well under way to determine whether HTLV-III and LAV represent the same virus.

HTLV-III has at this point been isolated in the Laboratory of Tumor Cell Biology at the National Cancer Institute over 80 times from over 50 individuals. This work has been a collaboration involving clinicians and epidemiologists within the National Institutes of Health and in several other institutions.[3] The virus can be recovered from over 85% of individuals with early AIDS and from roughly one third of patients with fulminant

[3] Collaborating clinical investigators include but are not limited to: Dr. Anthony Fauci, Dr. Henry Masur, Dr. Dan Longo, Dr. Bijan Safai, Dr. Jerome Groopman, Dr. James Goedert, Dr. James Curran, and Dr. William Blattner.

AIDS (5). These figures may be underestimates because the total of all AIDS specimens sent was analyzed even though some had substantially deteriorated en route. Moreover, there may be inherent difficulties in recovering live virus in late-stage AIDS because the $OKT4^+$ cell population thought to harbor the pathogen is so profoundly diminished. Technologies for detecting circulating antigens of HTLV-III as well as specific antigen-antibody complexes are being developed, and preliminary observations suggest that a high proportion of patients with AIDS have detectable antigenemia. Such tests may obviate some of the problems inherent in relying upon tests that depend on live virus isolation to monitor the in vivo replication of HTLV-III (C. Saxinger, M. Sarngadharin, and R. C. Gallo, unpublished).

Healthy heterosexual donors have never yielded virus (0 of 115 individuals tested), and the virus was isolated from only 1 of 22 control nonpromiscuous, homosexual men (from nonhigh risk regions of the USA) who appeared healthy. That one individual developed AIDS 6 months later (5).

In nearly 90% of AIDS patients it was possible to detect circulating antibodies reactive against HTLV-III (7). In more recent testing, using a combination of enzyme-linked immunosorbent and electroblot (Western) assays for detecting antibodies, 100% of AIDS patients were positive (79). Only one of 286 control individuals was positive. Moreover, seropositivity for HTLV-III can identify blood donors who had been suspected (on clinical and epidemiologic grounds) of transmitting AIDS via the transfusion of a blood product (B. Safai, M. Sarngadharan, R. C. Gallo; J. Groopman, M. Sarngadharan, R. C. Gallo, unpublished). Finally, in vitro infection of many T-cell populations by HTLV-III leads to their rapid destruction (S. Z. Salahudin, P. Markham, M. Popovic, and R. C. Gallo, unpublished).

Recent seroepidemiologic studies have cast a new light on the extent of the HTLV-III pandemic. The virus appears to be spreading in several parts of the world including (but not limited to) Austria, Central Africa, Denmark, Finland, Germany, Italy, Switzerland, and certain nations in the West Indies (R. C. Gallo, M. Robert-Guroff, M. Sarngadharan, W. Blattner, J. Goedert, C. Saxinger, and J. Schüpbach, unpublished).

Knowledge of HTLV-III may affect many phases of clinical investigation in AIDS. For example, the discovery offers new possibilities for early-case definition and risk assessment. One practical application is the imminent distribution of a rapid antibody-screening kit to prevent the donation of blood products by individuals who have been infected by the virus. The techniques for doing this are already at hand, and the Department of Health and Human Services has taken steps to make a nationwide system of serum testing available through a technology transfer to the private

sector. The availability of such tests is likely to pose a number of scientific and moral questions—at least in the short term. For example, what does one tell a seropositive health-care provider or a pregnant woman?

The discovery of an immunosuppressive retrovirus in AIDS might serve as a stimulus to reexamine endemic forms of cancer that pose special public health problems for some nations in the Third World. Could a subset of the endemic Kaposi's sarcomas and Burkitt's lymphomas of equatorial Africa be expressions of a subtle, retrovirus-associated immune abnormality?

The effect of more than one retrovirus infecting a given patient needs to be explored. Approximately 10% of patients with AIDS have evidence of infection involving yet another member of the HTLV family (usually HTLV-I, but sometimes HTLV-II, in addition to HTLV-III). Does infection with a combination of HTLV-family members affect the clinical presentation or course of AIDS? Do clusters of HTLV-viruses play a role in diseases other than adult T-cell leukemia and AIDS?

The current technology might make it possible to begin formulating rational interventions that are directed against a cause rather than a given manifestation of AIDS. In the future, it might be possible to develop a vaccine for individuals who belong to certain AIDS-risk groups and thereby prevent the disease. Moreover, strategies for restoring immune function by interrupting viral replication in AIDS or pre-AIDS patients (perhaps by using drugs that inhibit reverse transcriptase) or eliminating viral-bearing cells coupled with bone marrow transplantation might be developed based on an understanding of the biology of this new agent. Indeed, the availability of HTLV-III has made it possible to establish relatively rapid screening systems for pharmaceutical agents that can block replication of the virus. In this regard, a drug called suramin, which was shown in 1979 to inhibit the reverse transcriptases of animal retroviruses (80), has been shown to protect normal $OKT4^+$ (helper/inducer) T cells against the cytopathic effect of HTLV-III in vitro (H. Mitsuya, and S. Broder, unpublished), and also to block the infectivity and replication of the virus in a special neoplastic T-cell clone [H9] (M. Popovic, S. Broder, and R. C. Gallo, unpublished). This neoplastic T-cell clone is permissive for replication of HTLV-III without itself succumbing to the cytopathic effect of the virus, and can, therefore, be useful in providing a direct measure of HTLV-III replication in vitro. A pilot experimental trial of suramin to test whether it is possible to block in vivo replication of HTLV-III in patients with AIDS, and if so whether an improvement of the immunodeficiency state can be detected, is now under way at our institution.

In the near future, we believe a number of significant advances in medical oncology and clinical immunology will result from current research in the HTLV family.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 337–65

LAMBDA CHAINS AND GENES IN INBRED MICE

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INTRODUCTION

In inbred mice, immunoglobulins (Igs) with κ light (L) chains outnumber those with λ L chains by about 20:1 (1–3). A similar or even greater disproportion is found in mouse myeloma proteins (4). Therefore, amino acid sequence analyses of Ig L chains were initially carried out on κ chains. Many Vκ sequences, but only a single Cκ sequence, were revealed, in accord with later evidence for many $V\kappa$[1] but only a single $C\kappa$ gene segment (5, 6). Amino acid sequences of λ chains made by myeloma tumors suggested a similar arrangement, except that these sequences could be accounted for by only a single $V\lambda$ gene, as well as a single $C\lambda$ gene (7). The extremely limited sequence variability of the λ chains [see (7)] suggested, moreover, that they were not significant contributors to the enormous structural and functional diversity that characterizes murine Igs as a whole. While this viewpoint has not been altered by the subsequent finding of additional λ chain isotypes[2] (8–10), the overall theme of this review is that the very simplicity of the inbred mouse λ light-chain system offers special opportunities to learn about some fundamental properties of Igs, namely, structure-function relationships, Ig diversity, and regulation of Ig gene expression. This system may also turn out to contribute to our understanding of the T-cell antigen receptor, since recent findings point to significant structural homologies between gene segments for λ chains and for some receptor subunits (111, 112).

[1] In accord with convention, genes are italicized and their products are in roman type.

[2] Nomenclature: By convention the variant forms of an immunoglobulin chain are usually called isotypes when encoded by nonallelic genes. Accordingly, we refer to in this paper the three kinds of λ chains as isotypes, though elsewhere they are often called subtypes.

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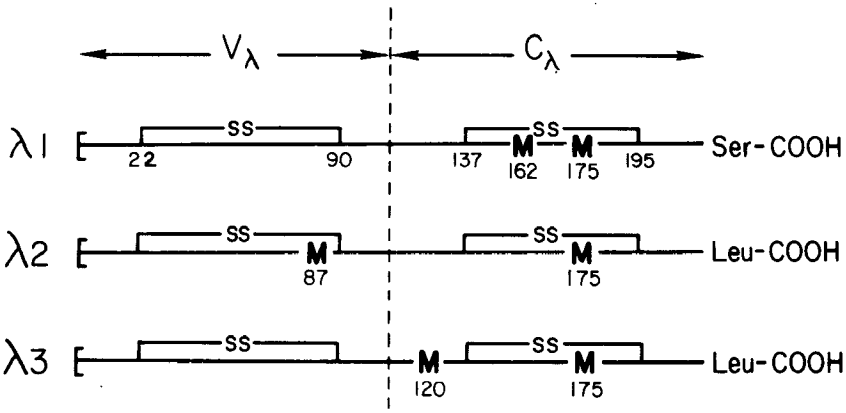

Figure 1 The distinctive positions of methionine residues (M) in each of the three λ chain isotypes. The positions of methionine and half-cystine residues are based on (8–11) and (20).

THE THREE λ CHAIN ISOTYPES

The most frequently encountered λ chain isotype (λ1) was found by analyzing the L chains produced by myeloma tumors that were unselected except for their production of λ1 chains (11–14). The second isotype (λ2) was revealed by the sequence of an L chain (8, 9) that was analyzed because it was from the first myeloma protein (MOPC-315) that was found to have high affinity for commonly studied ligands (DNP, 2,4-dinitrophenyl, and TNP, 2,4,6-trinitrophenyl) (15). Because of its similarity to λ2, the third type (λ3) was discovered (10) while analyzing L chains that were initially considered to be of the λ2 variety (16).

The three isotypes are compared diagrammatically in Figure 1. Each is defined by a distinctive C-region sequence (Figure 2) (9–11). Cλ2 and Cλ3 differ at only 5 out of 104 positions and have not been consistently distinguished serologically; however, they differ markedly from Cλ1 in sequence (Cλ2 at 39 and Cλ3 at 40 residues, see Figure 2) and they are easily distinguished from Cλ1 serologically.

The V region of each λ chain (positions 1–110),[3] like that of each κ chain, is encoded by separate *V* and *J* gene segments that become linked during B-cell development by a rearrangement that joins a *V* to a *J* segment (17). The

[3] Numbering system: All sequenced murine λ chains have the same number of V region amino acid residues. In this paper the positions are, accordingly, numbered sequentially, 1–110, from the amino-terminus to the end of the J region. The numbering is not interrupted by the letter designations introduced by Kabat and Wu (for residues in the first and third CDR) to maximize sequence homology of V regions of H, κ, and λ chains (41). As a consequence of this difference the λ chain positions designated here as 96, 97, 98, and 99 correspond to 94, 95, 96, and 97, respectively, in the Kabat-Wu numbering system (41).

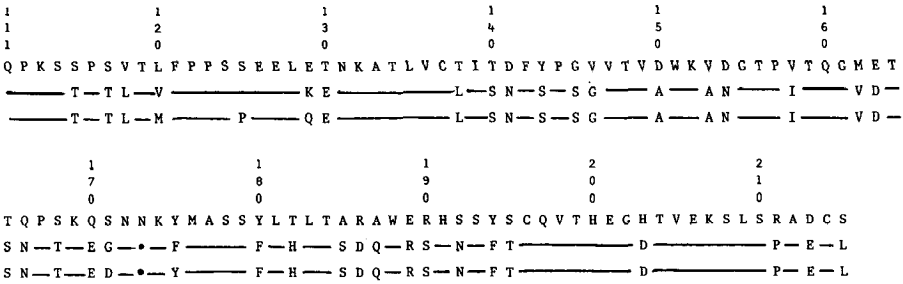

Figure 2 Amino acid sequences of the constant (C) regions of the three λ chain isotypes. The $\lambda 1$ constant region contains one more residue than the $\lambda 2$ and $\lambda 3$ constant regions. The difference is introduced at position 173 to maximize sequence homology (10). Amino acids are designated by the one-letter code. Same residue as $\lambda 1$: ——. Based on (8–11).

Vλ segments encode amino acid positions 1–96 and the *J*λ segments encode positions 99–110. Positions 97 and 98 mark the V/J boundary: As discussed below, nucleotides for the codons that specify the amino acids at these positions can be derived from *V*λ or *J*λ gene segments or both [see (18, 19)].

One of the differences between λ and other Ig chains (κ, H) arises from the invariant association of particular *J*λ and *C*λ segments. A hint of this invariance emerged when the first $\lambda 2$ and $\lambda 3$ chains sequences (9, 20) were compared with the previously determined sequence for $\lambda 1$ chains (11): The C region of each isotype appeared to be associated with a different J sequence (Figure 3). The significance of this association became evident when the genomic organization of λ gene segments was clarified.

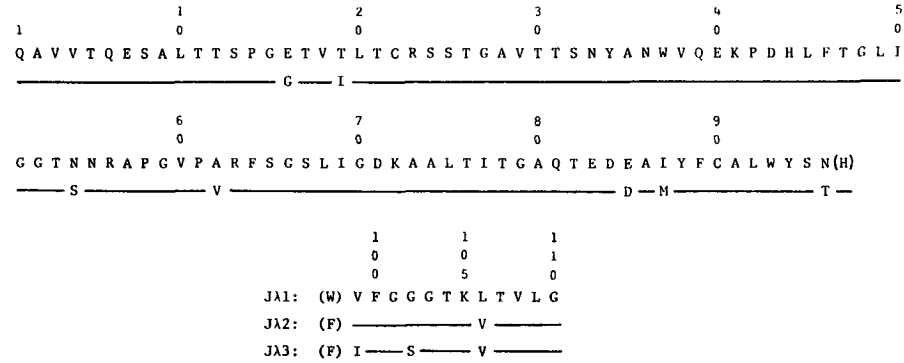

Figure 3 Amino acid sequences of the variable (V) regions of the three λ chain isotypes. The sequences shown correspond to the two germ line *V*λ gene segments (Vλ1, Vλ2) and to the three germ line *J*λ gene segments (Jλ1, Jλ2, Jλ3). Parentheses are placed around the amino acids at positions 97 (His) and 98 (Trp or Phe) to indicate that imprecision in joining *V* to *J* gene segments can result in various amino acid substitutions at these positions (see Figure 5). Same as Vλ1 or Jλ1 : ——. Based on (8–11, 18, 20, 21, 25, 26, 43, 110).

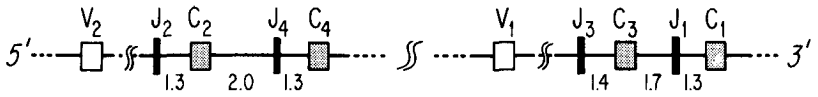

Figure 4 Organization of λ gene segments on chromosome 16 of the BALB/c mouse. The two clusters of *J-C* segments have not been linked physically to each other or to the *Vλ* gene segments. Distances between *Jλ* and *Cλ* segments are in kilobases. The basis for the 5′–3′ order is discussed in the text. Based on (23, 24, 29).

ORGANIZATION OF λ GENE SEGMENTS

Tonegawa et al discovered that there are two *Vλ* gene segments [*Vλ1*, *Vλ2*; see Figure 3 and (21, 22)]. Later, studies by Blomberg et al (23) and by Miller et al (24) showed that the *Jλ* and *Cλ* gene segments are distributed between two similar clusters, arranged as illustrated in Figure 4. Each cluster contains two *Cλ* gene segments, each separated on its 5′ side by a 1.3–1.4 1 kb (kilobase) intron from a unique *Jλ* gene segment. As a result there are four distinct *Jλ-Cλ* pairs. Since each λ isotype is defined by a unique *C* gene segment (Figure 2) and each *Cλ* segment is associated with a particular *Jλ* gene segment (Figures 3, 4), each λ isotype is also characterized by a distinctive J+C sequence, extending from position 99 to the carboxy terminus. The λ1, λ2, and λ3 isotypes are thus encoded, respectively, by the *Jλ1-Cλ1*, the *Jλ2-Cλ2*, and the *Jλ3-Cλ3* gene-segment pairs. The fourth pair, *Jλ4-Cλ4*, has several anomalies, suggesting that it is a pseudogene (25, 26). For example, *Jλ4* lacks a GT dinucleotide that occurs at codon 110 in all functional *J* gene segments and is an obligatory signal element for RNA splicing (27). *Jλ4* also has a 2 bp deletion from the heptamer sequence that serves as a signal for *V-J* rearrangement (-- CAGTG in *Jλ4* versus CACAGTG in *Jλ1*). These anomalies may explain why a λ4 chain has not been encountered and why even a nonproductive *Vλ* rearrangement to *Jλ4* has not been seen (28).

The finding that both rearranged and unrearranged forms of both *Vλ1* and *Vλ2* gene segments can occur in the same cell line [MOPC-315, and J558, (23)] led to the suggestion that *Vλ1* is upstream of the *J3C3-J1C1* cluster and *Vλ2* is upstream of the *J2C2-J4C4* cluster. The 5′–3′ order of these clusters is discussed below. Their localization on chromosome 16 has been demonstrated by Southern blot analyses of mouse-hamster hybrid cell lines (29).

V → J REARRANGEMENTS

A *Vλ* gene segment is not transcribed unless joined to a *Jλ* gene segment. Given two *Vλ* and four *Jλ* segments, eight V-J rearrangements are possible

(22, 23, 28, 30). However, in 74 λ chains whose *V-J* rearrangements were determined directly by Southern blot analyses of myelomas or hybridomas, or deduced from amino acid or cDNA sequences, only three of the eight possible rearrangements occurred regularly (V1-J1, V2-J2, V1-J3). Two occurred rarely (V2-J1, V2-J3), and three were not seen at all (V1-J2, V1-J4, V2-J4) (28). In this series, the *Vλ1* gene segment was recombined with *Jλ1* in nearly all (95%) λ1 chains and with *Jλ3* in nearly all (95%) λ3 chains, whereas *Vλ2* was recombined with *Jλ2* in all λ2 chains examined. In three rare hybridomas, *Vλ2* was recombined with *Jλ3* once (28, 31) and with *Jλ1* twice (28); in the resulting rare chains the encoding gene segments were *V2J3C3* or *V2J1C1*. These exceptions aside, the rule is that λ1 chains are encoded by *V1J1C1* segments, λ2 chains by *V2J2C2* segments, and λ3 chains by *V1J3C3* segments.

The sharply restricted pattern of *V-J* rearrangements suggests the gene order shown in Figure 4 (28, 31). Given this order, and the model for *V-J* rearrangement in which DNA between the recombined gene segments is deleted (32), it appears that when a λ-producing B-cell develops from a precursor pre-B cell, a *Vλ* gene segment will nearly always rearrange with a *Jλ* gene segment in the nearest downstream *J-C* cluster. Rarely, *Vλ2* rearranges to a *Jλ* segment in the distant cluster, but rearrangement in the upstream direction (*V1-J2*) has not been seen and may not be possible.

Since there is virtually only one *V-J* rearrangement per λ chain isotype, *V-J* combinatorial variation contributes almost nothing to the diversity of λ chains, unlike its role in the diversity of κ and H chains (32–35). With κ, for example, there are four functional *Jκ* (32, 34) and on the order of 200 *Vκ* gene segments (17, 36, 37). If their rearrangements are unrestricted, as is generally believed, there would be 800 Vκ-Jκ combinatorial variants; with H chains the number of such variants must be far greater, because as many as 10 to 20 *DH* segments are additionally recombined in forming *VH-DH-JH* assemblies (38–40).

V/J JUNCTIONAL VARIANTS

Combinatorial variation in the assembly of gene segments (*V-J*, *V-D-J*) can contribute to diversity of V region primary structure not only directly, but also indirectly, because imprecision in assembling the segments can create novel codons at the joining sites. Such V/J junctional variants have been described for κ chains (32, 34, 35). Do they also occur with λ chains?

Until recently (19), all sequenced λ chains had tryptophan (Trp) at position 98 of λ1 chains (7, 41), phenylalanine (Phe) at position 98 of λ2 chains (42); and histidine (His) at position 97, regardless of λ isotype (41, 42). It seemed, thus, that λ chains might lack V/J junctional diversity (as well as V-J

combinatorial variability, see above). However, nucleotide sequences at the 3′ end of unrearranged $V\lambda$ gene segments and at the 5′ end of unrearranged $J\lambda$ gene segments suggest that in-phase $V\lambda$-$J\lambda$ recombinations can lead to various amino acids at the V/J boundary. Shown here in Figure 5 are three possibilities (Trp, Leu, Phe) at position 98 of λ1 chains, two (Phe, Tyr) at position 98 of λ2 chains, and two (His, Gln) at position 97 of λ3 chains. Of

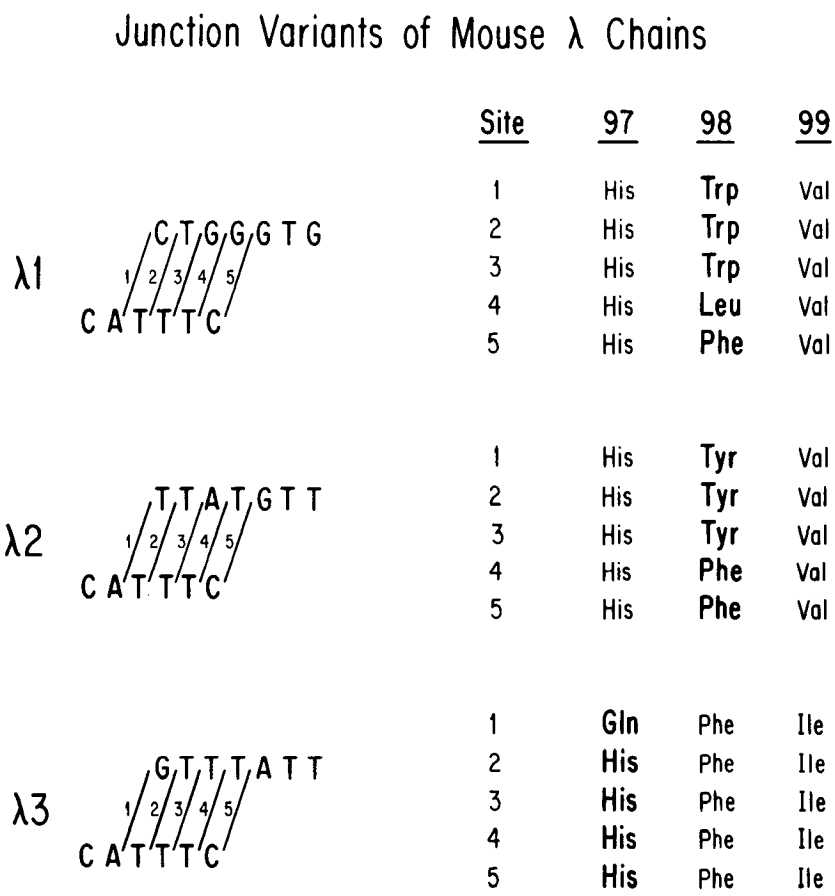

Figure 5 Variation in sites for recombination between $V\lambda$ and $J\lambda$ gene segments. A different combination of segments is utilized for each of the three λ isotypes. For λ1, λ2, and λ3 the recombining segments are (rare exceptions aside), Vλ1-Jλ1, Vλ2-Jλ2, and Vλ1-Jλ3. For each recombining pair shown, the V segment is below and to the left, the J segment is above and to the right; the lines joining them, numbered 1–5, refer to alternative, in-phase recombination sites that link the 3′ end of the *V* segment to the 5′ end of the *J* segment. (Each short sequence is written with 5′ at the left and 3′ at the right.) The resulting alternative amino acids at positions 97–98 are listed at the right bold face. All of the substitutions except Phe at λ1 98 have been observed (18, 19, and footnote 4). The sequences are from (21, 25, 26, 43).

these seven possibilities, six have been detected in recent studies that focussed on λ chains from Igs with unusual ligand binding activity (see STRUCTURE-FUNCTION RELATIONSHIPS). In these studies, a $\lambda 2$ chain with Tyr at position 98 and a $\lambda 3$ chain with Gln at position 97 were identified (18, 19, 42). In an analysis of λ chains from anti-DNP monoclonal antibodies (mAb) from hyperimmunized mice, $\lambda 1$ chains with Leu at position 98, in place of Trp, have also been identified (K. Tamoto, E. B. Reilly, T. Azuma, H. N. Eisen, manuscript in preparation). All of these substitutions at positions 97 ($\lambda 3$) and 98 ($\lambda 1$, $\lambda 2$) can be accounted for by variability in the precise internucleotide site of *V-J* recombination (Figure 5). Somatic mutation cannot be totally ruled out as an alternative explanation but it is most unlikely, especially when a chain's entire V+J sequence corresponds precisely to the germ-line sequence of the $V\lambda$ and $J\lambda$ gene segments that encode that chain; such a correspondence has been found for all six of the V/J junctional variants identified (18, 19). The special functional significance of amino acid substitutions at the V/J junction is discussed below (see STRUCTURE-FUNCTION RELATIONSHIPS).

V REGION SOMATIC MUTATIONS

Nucleotide or amino acid differences between a germ-line sequence and its expressed V+J sequence are defined as a V region somatic mutation.[4] Therefore, to establish that a somatic mutation has occurred requires that the sequence of the particular germ line *V* and *J* segments that encode an expressed V+J sequence be known. Since the nucleotide sequences of all germ line $V\lambda$ and $J\lambda$ gene segments have been established for the BALB/c mouse (21, 25, 26, 43), and the segments that encode any particular λ chain are readily identifiable, the recognition of somatic mutations is straightforward from the $V+J$ sequences of λ chains of BALB/c origin. (For κ and H chains, however, there are many similar $V\kappa$ and many similar VH gene segments; thus it is not easy to identify with certainty the germ line V segments that correspond to V segments that encode particular κ and H chains.)

In an extensive study of V regions of $\lambda 1$ chains, Weigert & Cohn and their colleagues found that 12 of 18 chains had the same V region sequence and that in the other 6 amino acid replacements totalled 7–9 [Figure 6 and (7)]. They correctly inferred that the identical repeats represented the sequence of a $V\lambda$ germ line gene; thus the frequency of V region amino acid replacements (i.e. somatic mutations) in this series of $\lambda 1$ chains was only 0.5

[4] It is formally possible that what appears to be a somatic mutation could be a germ line mutation, i.e. a difference between the consensus germ line sequence for the species or strain and the germ line of the particular animal that produces the expressed V+J sequence.

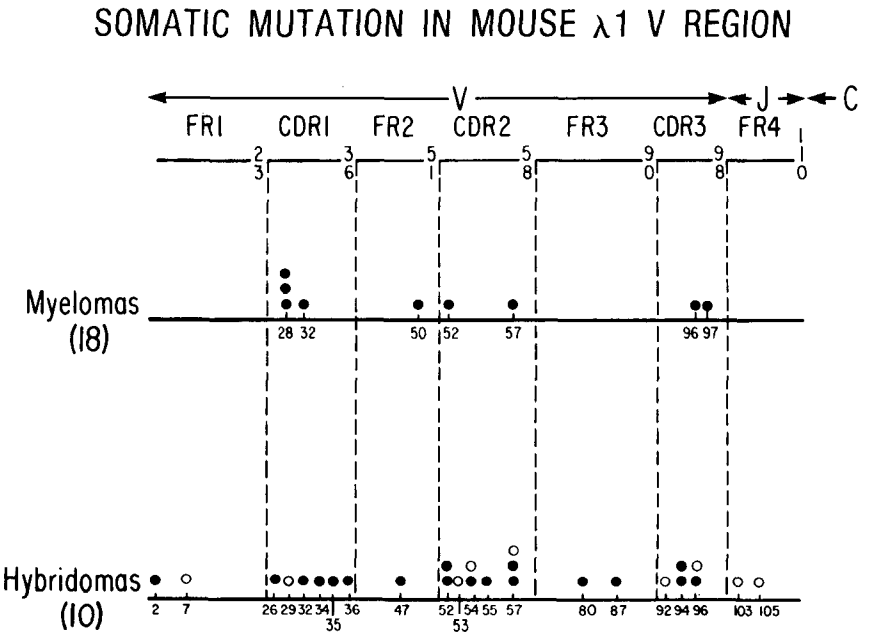

Figure 6 Somatic mutations in V regions of $\lambda 1$ chains. The positions of mutations are shown in chains from 19 myeloma proteins (*top*), analyzed by amino acid sequencing (7), and from 10 monoclonal antibodies (*bottom*) determined by sequencing cDNA from one hybridoma making an IgG2a anti-NP antibody (113), two making IgG1 anti-dansyl and seven making IgG1 anti-DNP antibodies (K. Tamoto, E. B. Reilly, T. Azuma, H. N. Eisen, in preparation). ○, silent mutations, ●, replacement mutations. The V/J junction residues at positions 97 and 98 are not shown.

(or less) per chain. A similar frequency probably exists among $\lambda 2$ chains: In one set of four such chains (42) there was only one V region replacement mutation (0.25 per chain), whereas in another $\lambda 2$ chain [from a myeloma protein with high affinity for DNP ligands (15)] four V-region mutations were found (9), bringing the total for the five $\lambda 2$ chains to five amino acid replacements.

To determine whether the frequency of V region somatic mutations is higher in antibodies from intensively immunized mice than in myeloma proteins, Tamoto et al recently analyzed V regions of $\lambda 1$ chains from hybridomas making monoclonal antibodies (anti-DNP) of the IgGl class (K. Tamoto, E. B. Reilly, T. Azuma, H. N. Eisen, manuscript in preparation). The choice of these hybridomas was based upon two considerations: (*a*) that the frequency of V region somatic mutations is higher in γ than in μ chains (38, 44, 45–48) and (*b*) that when somatic mutations occur in a B-cell's H chain they are also likely to occur in the V region of the same cell's L

chain. Accordingly, the cDNA from $\lambda 1$ mRNA was sequenced [by the primer extension method (18)], using as a source of mRNA the hybridomas that were derived from hyperimmunized BALB/c mice and were making IgGl, $\lambda 1$ monoclonal antibodies (K. Tamoto, E. B. Reilly, T. Azuma, H. N. Eisen, manuscript in preparation). The V region of 10 of these $\lambda 1$ chains had 18 replacement somatic mutations, or an average of 1.8 per chain (Figure 6). There were also nine "silent" mutations, bringing the average to a total of 2.7 mutations per chain. That one third were silent is expected from the genetic code. The higher frequency of replacement mutations found in this study than in the others probably reflects selection by antigen during hyperimmunization of particular B cells, probably those producing Igs with high affinity for the DNP group (49).

The Mutation Frequency in Complementarity-Determining Versus Framework Regions

The distribution of mutations between complementarity determining (CDR) and framework regions (FR) also suggests that selective pressures influence the nature and frequency of the mutations identified in Igs. In V regions of the sequenced κ chains this distinction is not so clear, as mutations seem to be almost as frequent in FR as in CDR (50–55). However, in the study by Tamoto et al of $\lambda 1$ chains from IgGl mAbs derived from hyperimmunized mice, the frequency of replacement mutations was about 20 times higher in CDRs than in FRs (Figure 6) (K. Tamoto, E. B. Reilly, T. Azuma, H. N. Eisen, manuscript in preparation). The low frequency of replacement mutations in FRs suggests that there is strong selection against amino acid substitutions in these regions, probably to conserve their secondary structure and preserve the L-H chain interactions needed to maintain the integrity of the Ig molecule's architecture and its combining sites. In contrast, the high frequency of replacement mutations in CDRs (20 in 270 or almost 1 in 10 codons, see Figure 6) suggests that in these regions there are very few constraints; indeed, CDR mutations that result in better binding sites and higher affinity are probably selected for by antigen.

Number of Primary Structure Variants of $\lambda 1$ Chains

How many V region variants are there among the three λ chain isotypes? *V*$\lambda 1$ can recombine with *J*$\lambda 1$ and (rarely) with *J*$\lambda 3$, and each of these rearrangements can result in 2 or 3 V/J junctional variants (neglecting a third possibility, Phe, that has so far not been seen; Figure 5). There are thus 4 or 5 $\lambda 1$ and $\lambda 3$ V/J variants; similarly there are two $\lambda 2$ V/J variants, each resulting from the *V*$\lambda 2$-*J*$\lambda 2$ rearrangement. There are also four additional very rare variants due to the unusual *V2-J1* and *V2-J3* rearrangements and

the probability that each of these is also associated with two V/J junctional variants (Figure 5). Hence, altogether, in the absence of somatic mutations, 11 primary structure V region variants are possible: Eight of these have been detected, but three of the 11 probably account for nearly all of the λ chains having germline (i.e. nonmutated) V region sequences: $\lambda 1$ chains having Trp at position 98; $\lambda 2$ having Phe at position 98, and $\lambda 3$ having His at position 97).

However, the contributions of V/J junctional variation (and of V/J combinatorial variation) to λ chain diversity is almost insignificant when compared to the effects of somatic mutation. In calculating the contribution of somatic mutation to diversity we consider that: (*a*) The average single base change that leads to an amino acid replacement can occur in the first or second position of a codon and result (on average) in six alternative replacements for the amino acid at that position in the germline sequence; (*b*) these replacement mutations are largely limited to the 27 positions in the three CDRs, since elsewhere in the V region (i.e. in FRs) they are likely to result in an unstable Ig structure; (*c*) the replacement mutations can probably occur in any CDR position, and in these positions there are very likely to be no forbidden substitutions; (*d*) fluctuations in the number of mutations per chain can be estimated from the Poisson distribution. To emphasize the last point we note that under conditions where there are, on the average, 1.8 replacement somatic mutations per $\lambda 1$ chain (see Figure 6), 16% of the chains will have three such mutations, 7% will have four, etc.

Since there are $n!/(n-x)!x!$ different combinations of x replacement mutations in a chain having n susceptible codons, the number (N) of different amino acid sequences is:

$$a^x \cdot \frac{n!}{(n-x)!x!},$$

where a is the number of alternative amino acids resulting from a single base change in the first or second position of a mutated codon, x is the number of such mutated codons per chain, and n is the number of codons that are susceptible to these replacement mutations. For reasons given above, we assume $a = 6$ and $n = 27$. Hence, when x is 0, 1, 2, 3, 4 the corresponding number of chains with different V region amino acid sequences is (respectively) 1, 162, 1.26×10^4, 6.26×10^5, and 2.28×10^7. To account for V/J junction variation each of these numbers should be multiplied by two or three (see Figure 5).

From the findings of Tamoto et al and the foregoing estimates it follows that in BALB/c mice the number of different V-region sequence variants of just this one isotype ($\lambda 1$) is close to and probably exceeds the total number of B-cell clones in the individual mouse. Hence the size of the potential Ig

repertoire in an individual is probably fixed not by its ability to generate Igs with a vast number of V region sequences but rather by the total number of B-cell clones it has and the rate at which these clones turn over, i.e. are replaced by new clones, arising from precursor cells.

STRUCTURE-FUNCTION RELATIONSHIPS

A central dogma of immunology is that amino acid sequences in CDRs determine an antibody molecule's specificity and affinity for ligand. However, the application of this generalization to particular sequences and ligands has been almost impossible, and the general rules that govern the connection between particular primary structures and ligand-binding activities remain almost totally obscure.

Primary Structure and Affinity

Recently, Azuma et al (19) have taken advantage of some currently available λ chains that differ from each other in sequence by only one or a few amino acids to ask whether these few differences affect ligand-binding activity. To answer the question they recombined each of these chains with the H chain (H^{315}) from an anti-DNP myeloma protein (M315) and thereby obtained a set of reconstituted Igs that also differed in sequence by just one or a few amino acids at known positions in the L chains. Differences in affinity of these reconstituted Igs for DNP ligands, summarized in Figure 7, revealed a surprisingly pronounced effect of amino acids at the V/J junction (positions 97 and 98 in λ chains).

In a striking demonstration of this junction effect, two reconstituted Igs (H^{315}-L^{8-13} and H^{315}-L^{5-7}) that differed almost 1000-fold in affinity for DNP aminocaproate differed in primary structure by only a single phenylalanine (Phe)-tyrosine (Tyr) substitution at the V/J junction of their L ($\lambda 2$) chains (see Figures 8 and 5), i.e. by only one out of approximately 660 amino acids (L + H chains), and, indeed, by only the single O atom difference between Phe and Tyr. In another example, a pair of reconstituted Igs (H^{315}-L^{5-8} and H^{315}-L^{C49}) that differed in sequence by only a glutamine (Gln)-histidine (His) substitution at the V/J junction of their L ($\lambda 3$) chains (see Figures 8 and 5) also differed in affinity, though only four-fold. If the V/J junction effect proves to be a general one, it would mean that gene segment assembly (V/J in L chains and perhaps also V/D/J in H chains) is an important source not only of primary sequence variation (as is well known) but also of ligand-binding diversity.

If the effect of the V/J junction residues is due to VL interactions with VH, rather than to direct interactions with the bound ligand itself, the impact of a particular residue at the λ chain junction must also depend upon the VH

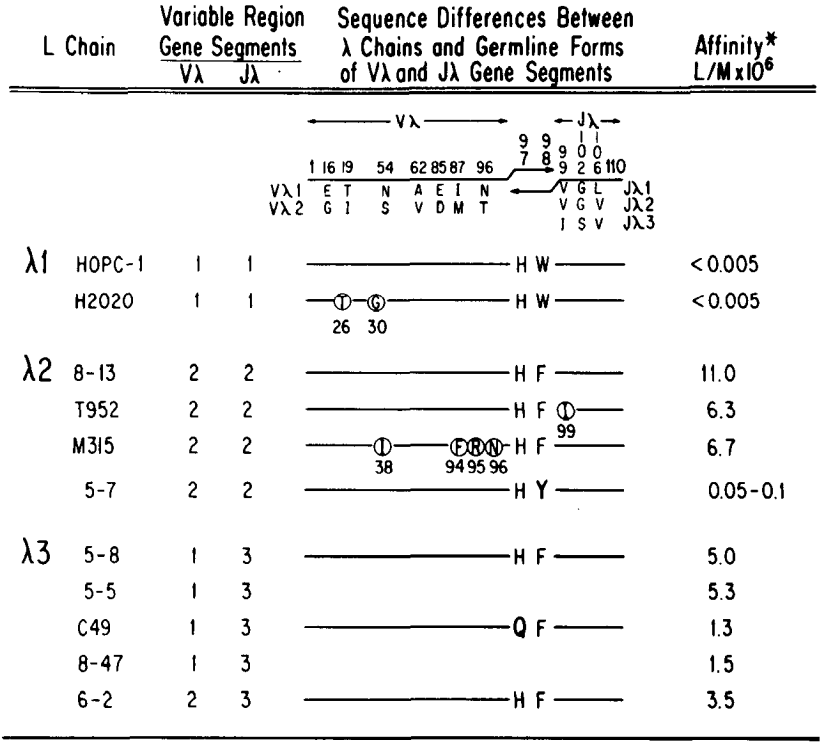

Figure 7 Correlation between V region sequences of λ chains and affinity for ε-DNP-aminocaproate of the reconstituted Igs made with each of these chains and H^{315}, the heavy chain from an anti-DNP myeloma protein (MOPC-315). Circled amino acid residues indicate differences from the germ line sequences. Comparison of germ line sequences at the top of the Figure shows only those positions and residues at which the *Vλ1* and *Vλ2* and the *Jλ1*, *Jλ2*, and *Jλ3* gene segments differ. From Azuma et al (19).

sequence of the paired H chain. This argument is supported by the observation that although λ chains with junctional Phe, and not with junctional Tyr or tryptophan (Trp), form good DNP-binding sites with H^{315} (see Figure 7), in other Igs, having other H chains, λ chains with Tyr or Trp at the junctional position can be present in Igs with high-affinity anti-DNP sites (for example, the antibody from which L^{5-7} was isolated; also K. Tamoto et al, in preparation).

In contrast to the striking effect of substitutions at the V-J boundary, amino acid substitutions elsewhere in the V region, whether in framework (FR) or complementarity-determining regions (CDR), had little effect on affinity for DNP ligands of the reconstituted Igs referred to in Figure 7. For

instance, a pair of reconstituted Igs (H^{315}-L^{315} and H^{315}-L^{952}) that differed at five V region positions, including three in the third CDR, had essentially the same affinity (6.3×10^6 vs 6.7×10^6 L/M). The lack of effect of these substitutions on affinity for ε-DNP-aminocaproate could mean that this hapten was an inappropriate ligand, perhaps because it is too small (56), or because a DNP-like antigen was not involved (in vivo) in the development of the B cells that gave rise to the myeloma tumors (MOPC-315 and TEPC-952) from which these L chains were derived. This last possibility emphasizes the need for what might be called prospective rather than retrospective analyses. The work of Azuma et al (19), summarized in Figure 7, represents a retrospective study: Myeloma proteins and monoclonal antibodies that happened to be available were used to furnish the chains for analysis of structure-function relationships with a ligand (DNP) that had no clear relationship to the antigenic stimulus that elicited the production of most of these Igs. It may prove more informative to carry out future studies of the type illustrated in Figure 7 with chains from monoclonal antibodies that have been deliberately elicited by an antigen that resembles the ligand used to analyze the reconstituted Igs.

Some λ chains, in the absence of H chains, bind to ε-DNP-lysine Sepharose beads (19). Free λ chains are predominantly dimers in solution and the L^{315} dimer is known to bind DNP-lysine (57, 58), though with several orders of magnitude lower affinity than the corresponding intact Ig (M315) or reconstituted Ig (H^{315}-L^{315}; see Figure 7). In general, the binding of various L chains to ε-DNP-lysine beads paralleled differences in affinity of the reconstituted Igs formed by the same L chains with H^{315} (19).

Primary Structure and Idiotypy

Because of their limited sequence diversity, λ chains are also useful for studying the relationship between primary structure and idiotypy. To illustrate this point, consider the reconstituted Igs made with H^{315} and the λ chains shown in Figure 7. These Igs were tested for ability to block the binding of ^{125}I-M315 by antibodies to the idiotype (Id) of myeloma protein 315 (M315). (The anti-Id antibodies were produced by immunizing BALB/c mice with M315, a protein of BALB/c origin; the resulting isologous anti-Id antibodies reacted only with M315, not with other myeloma proteins (59, 60).) Only the homologous reconstituted Ig (H^{315}-L^{315}) blocked this reaction, and it did so completely and as well as native M315 in inhibition-titration assays; the other reconstituted Igs, shown in Figure 7, were without any effect (T. Azuma, V. Igras, H. N. Eisen, manuscript in preparation).

What distinguishes the primary structure of L^{315} from that of other λ2 chains are the four circled amino acids shown in Figure 7. One, a

conservative substitution in the second FR, is probably insignificant and it is very likely that the three contiguous substitutions in the third CDR, Phe-Arg-Asn, are required for the Id that is recognized by isologous anti-315 Id antibodies.

Idiotypic determinants recognized by anti-Id antibodies depend not only on L, but also on H chains (59). Thus, reconstituted Igs made with L^{315} and H chains other than H^{315} also do not react with isologous anti-M315 (Id); therefore this Id is defined by some as-yet-unknown sequence in the V region of H^{315}, in addition to the third CDR Phe-Arg-Asn of L^{315}.

The isologous anti-Id responses elicited by myeloma protein M315 in BALB/c mice involve not only antibodies but also T cells. Some of these cells cause a delayed type hypersensitivity (DTH) reaction and others seem to suppress this reaction (66). Unlike the isologous anti-315 Id antibodies, the isologous anti-315 Id T cells seem to react with L^{315} alone, in absence of H chains, but they do not react with other λ chains. Very likely, therefore, the amino acids that are necessary, and perhaps sufficient, for recognition by the isologous anti-315 Id T cells are also the three contiguous products of somatic mutation in the third CDR of L315 [Phe-Arg-Asn, Figure 7; (66)].

FREQUENCY OF λ CHAINS AND λ ISOTYPES

λ-B Cells vs κ-B Cells

In accord with the much greater abundance in serum of κ-Igs than λ-Igs, κ-producing B cells are over 20-times more numerous than λ-producers in LPS-stimulated spleen cells (67–69). Interestingly, however, in similarly stimulated bone marrow cells κ-producers are only about four times more frequent than λ-producers (69). In bone marrow the ratio of κ-:λ-B cells (4:1) probably represents B cells that have just arisen from pre-B cells, whereas in spleen the ratio ($>20:1$) probably reflects antigen-selection of mature B cells, with greater stimulation of κ-B than λ-B cells by randomly encountered antigens, because V region diversity of surface Igs of the κ-B cell population must greatly exceed that of the λ-B cell population.

It would be useful to know the frequency in bone marrow of B cells that produce each of the λ isotypes. In the absence of this information the frequencies of the λ isotypes in Igs and in B cells, described below, probably reflect the effects of antigen-selection, as much as (or perhaps even more than) the intrinsic frequencies in virgin B cells.

Normal Serum Ig

In sexually mature mice the average serum concentrations of Igs with $\lambda 2$ chains ($\lambda 2$-Igs) plus those with $\lambda 3$ chains ($\lambda 3$-Igs) varied among the inbred

strains from a high of about 80–140 μg/ml in the AL/N, NZB, BALB/c strains to a low of about 15–25 μg/ml in the BSVS, C58/J, CE/J, SJL/J, DBA/2, and C3H/He strains (70). The differences did not correlate with Ig heavy chain allotypes or major histocompatibility complex haplotype. In BALB/c mice λ1-Igs are about four times more frequent than λ2-Igs (3), and the latter are probably somewhat more frequent than λ3-Igs (71). Based on these relationships, serum concentrations of λ1-Igs are calculated to be about 300–400 μg/ml, corresponding to an estimated total serum Ig concentration of about 7 mg/ml. In SJL mice (see below), which virtually lack λ1-Igs (72, 73), the level of λ2-Igs (and probably total λ-Ig) is only about 25 μg/ml (70). In mass terms, therefore, λ-Ig represents an almost negligible proportion of total normal serum Ig.

In Antibodies Elicited By Certain Antigens

Despite the low frequency of λ-Ig in normal serum and the paucity of $V\lambda$ genes, certain antigens elicit antibodies that contain a high proportion of λ chains. Two that are known to evoke such responses are the 4-hydroxy-3-nitrophenylacetyl (NP) group (74) and dextrans having an alternating (1–3),(1–6) backbone (75, 76). It has recently been found that high λ-antibody responses are also elicited by the 2,4-dinitrophenyl (DNP) group (77) and by the 5-dimethylaminoapthalene-1-sulfonyl (dansyl) group (K. Tamoto et al, in preparation).

In the primary response to NP-protein conjugates about 90% of the antibodies made in C57BL/6 mice have λ1 chains (74; and T. Imanishi-Kari, personal communication) and in BALB/c mice about 50% have λ1 chains (78). Thereza Imanishi-Kari has also shown, more strikingly, that λ1 chains are present in 49 out of 50 anti-NP monoclonal antibodies from hybridomas generated by primary immunization of C57BL/6 mice; the remaining one had a λ3 chain (T. Imanishi-Kari, personal communication). A useful way to analyze the comparative frequency of λ isotypes in antibodies is illustrated in Figure 8. ^{35}S-methionine-labelled polyclonal anti-NP antibodies made by spleen cells from C57BL/6 mice (immunized with NP-chicken γ globulin) also reveal the great predominance of λ1 over the other isotypes (77).

In the response of BALB/c mice to dextran with the alternating (1–6),(1–3) backbone nearly all the antibodies contain λ chains (75, 76). Their subtypes have not been thoroughly characterized, but from one preliminary observation they seem to be almost exclusively λ1 (V. Igras, personal communication).

A recent study of the L chains of anti-dansyl antibodies by Fan & Karush (79) together with Tamoto, Reilly, Eisen (in preparation) has also revealed in this response a surprisingly high proportion of λ chains. The anti-dansyl

molecules made in BALB/c mice against dansyl-ficoll and in primary and secondary responses against dansyl-bovine γ globulin have L chains that are mainly $\lambda 1$, but the predominance of this isotype differs somewhat in IgM and IgG molecules. In the IgM anti-dansyl monoclonal antibodies, about 80% had λ chains, but in this set $\lambda 1$ was no more frequent than the sum of $\lambda 2$ and $\lambda 3$. In the corresponding IgG antibodies, about 95% had λ chains and of these about 50% were $\lambda 1$, 33% were $\lambda 2$, but none was $\lambda 3$. The excess of $\lambda 1$ over $\lambda 3$ is especially striking and is commented on below.

In antibodies made against the DNP group (another response recently shown to have a high proportion of λ-antibodies (77)), about 40% of the polyclonal anti-DNP antibodies made in primary and secondary responses of BALB/c mice to DNP-chicken γ globulin have λ chains (Figure 9) (77). In 60% of these antibodies the isotype is $\lambda 1$; the other 40% seem to be divided about equally between $\lambda 2$ and $\lambda 3$. Thus in BALB/c anti-DNP molecules $\lambda 1$ is only about three times more frequent than either of the other λ isotypes. Similar results have been found in (77): (*a*) culture supernatants from about

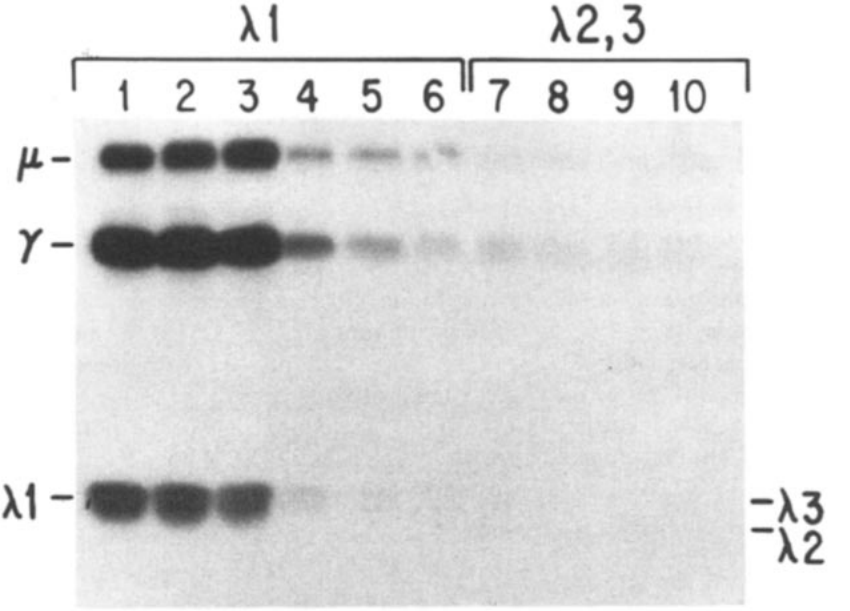

Figure 8 λ chain isotypes in anti-NP antibodies. ^{35}S-methionine-labeled antibodies, synthesized by spleen cells from BALB/c mice immunized with NP-chicken γ globulin were purified, immunoprecipitated sequentially six times by antibodies to $\lambda 1$ and then four times by antibodies to the other isotypes and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Note that $\lambda 1$ chains are abundant whereas $\lambda 2$ and $\lambda 3$ chains are virtually undetectable. From (77).

200 hybridomas, and (*b*) the ^{35}S-methionine labeled antibodies secreted by spleen cells (Figure 9). Similar frequencies have also been found in the predominantly IgM anti-DNP monoclonal antibodies elicited by DNP-ficoll (80).

It is not clear why λ chains are so prominent in the response to certain antigenic groups. There are no obvious chemical features that distinguish the antigens that elicit predominantly λ-Abs. For instance, one of these is the anionic form of NP; others are hydrophobic and nonionic, and one is a polysaccharide [dextran with the alternating (1,3),(1,6) backbone].

Why is λ1 the dominant isotype? The reasons for the predominance of λ1 over the other λ isotypes in serum Igs and in antibodies to NP, DNP, dansyl, and dextran are also not known. The very large difference between λ1 and λ3 is particularly puzzling, because in the functional (i.e. rearranged) gene for nearly all chains of both isotypes the same Vλ1 gene segment is used (28). Hence, the rearranged λ1 and λ3 genes have not only the same V region sequence from positions 1–98 (ignoring somatic mutations) but also

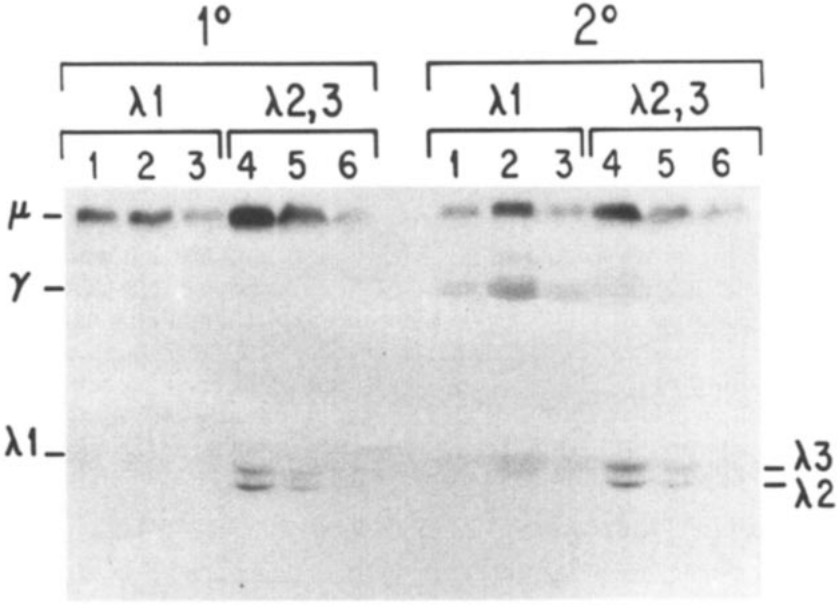

Figure 9 λ chain isotypes in anti-DNP antibodies. ^{35}S-methionine-labeled antibodies, synthesized by spleen cells from BALB/c mice immunized with DNP-chicken γ globulin, were purified after primary, 1°, and secondary, 2°, immunizations and analyzed as described in Figure 8. Note that in these antibodies λ2 and λ3 chains are relatively abundant: λ1 chains are actually slightly more abundant but appear less so here, perhaps because they migrate as less compact bands than the λ2 and λ3 chains. From (77).

the same upstream noncoding sequences for promoter(s) and possible enhancers. Moreover they can have the same V/J junctional residues (Figure 5).

Several possible explanations for the λ1 predominance have been considered. One ("antigen-selection") is that the antibodies formed by particular H chains and λ1 chains might have higher affinity for an antigen than the corresponding molecules formed with λ2 or λ3 chains. However, this explanation seems not to be valid, because no significant differences in affinity for NP-ligands were observed for reconstituted Ig molecules made by recombining various λ1, λ2, and λ3 chains with the H chains isolated from λ1-containing anti-NP monoclonal antibodies (81). Moreover, the reconstituted molecules made with λ2 or λ3 chains reacted with antibody to the NP^b Id, the characteristic idiotype of λ1-containing anti-NP antibodies from C57BL/6 mice (81). This limited observation suggests that idiotype regulation is probably also not responsible for the λ1 predominance.

Isotype-specific regulatory T cells, that might enhance responses of λ1-B cells or suppress responses of λ2-B cells and λ3-B cells also do not seem to be responsible for the markedly different λ-isotype frequencies in the anti-NP and anti-DNP responses. Though λ1 is the predominant L chain isotype in both anti-NP and anti-DNP antibodies, the frequency of λ2 and λ3 chains is greater in anti-DNP than in anti-NP antibodies. Thus, the ratio of $\lambda 1/\lambda 2+\lambda 3$ in anti-NP is about 30, whereas in anti-DNP it is about 1.5 (77). This large difference was consistently observed in BALB/c (and C57BL/6) mice injected with NP-chicken γ globulin (to elicit anti-NP antibodies) or DNP-chicken γ globulin (to elicit anti-DNP antibodies). If regulatory T cells were responsible for the isotype disparity between the anti-NP and anti-DNP responses, the injection of a mixture of the two antigens (NP-CGG and DNP-CGG) would be expected to lead either to preferential stimulation of λ1-producing B cells or preferential suppression of λ2- or λ3-producing B cells, with the result that the $\lambda 1/\lambda 2+\lambda 3$ ratio in the resulting anti-DNP molecules would come to resemble the ratio in the anti-NP Abs (or vice versa). However, the ratios were unaffected by immunizing the mice with either antigen alone or with a mixture of the two (77).

In Normal (Resting) Spleen B Cells

The predominance of Igs with λ1 chains over those with λ2 or λ3 chains would be understandable if λ1-B cells were much more numerous than λ2- or λ3-B cells. However, immunofluorescence has indicated that λ1- and λ2-B cells have nearly the same frequency in fetal liver and in normal spleen (83). Because the test antiserum probably did not distinguish between λ2 and λ3 this meant that the frequency of λ1-B cells equaled the sum of frequencies of λ2- plus λ3-B cells. In agreement, the ratio of ^{35}S-labeled λ1,

λ2, and λ3 chains synthesized by normal spleen cells, measured by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, was indeed subsequently found to be about 1:0.7:0.3 (71). Virtually all λ chains have two methionine residues per chain (Figure 1), and the rate of incorporation of ^{35}S-methionine into the various λ isotypes was roughly proportional to the frequency of the corresponding B cells. Hence, it is probable that the rate of λ chain synthesis is the same per resting B cell, regardless of the isotype it produces.

The small differences in frequency of various λ-B cells appear not to be due to selective effects of environmental antigens or regulatory T cells, since the same ratios were found in adult spleen and in neonatal and nude mouse spleen (71). It is possible that these differences arise during B-cell development from different probabilities in the V-J joining reaction for the different isotypes. Different heptamer signal sequences at the 5′ end of the three functional *Jλ* gene segments could result in *V-J* rearrangements being more probable for *Vλ1-Jλ1* than for *Vλ2-Jλ2* and least probable for *Vλ1-Jλ3* (25, 26).

In Polyclonally Stimulated Spleen Cells

Despite the small differences in frequency of the various λ-B cells in normal (resting) spleen, when these cells are stimulated by mitogens they produce greatly disparate amounts of λ chain isotypes, more in accord with the frequencies in serum Ig and in various antibodies. In LPS (or 8-bromoguanosine)-stimulated spleen cells, λ1 chains were synthesized 7 times faster than λ2 chains and 10 times faster than λ3 chains (82). When these rates were corrected for the relative frequencies of the respective B cells (which are the same in LPS-stimulated and resting spleen cells) λ1 chains appeared to be synthesized five times faster per stimulated cell than λ2 or λ3 chains; the latter two were made at about the same rate per cell (82).

Are there λ gene enhancer sequences? Why should λ1 chains be produced faster (per B-cell blast) than each of the other λ chains? A possible explanation is suggested by sequence homologies. As noted before, functional (i.e. rearranged) *λ1* and *λ3* genes nearly always have the same *Vλ* gene segment (*Vλ1*) (28) and therefore the same noncoding upstream sequences (for promoters, etc). However, downstream of their respective *V* and *J* gene segments, the rearranged *λ2* and *λ3* genes are very much alike and both differ markedly from *λ1* in *C* gene segments (see Figure 2), probably in the noncoding *J-C* introns (25), and perhaps also in untranslated sequences downstream of the *C* segments. It is possible, therefore, that regulatory sequences in untranslated regions that flank the *C* gene segments (or perhaps even in this segment itself) are responsible for the disparate rates of expression of the rearranged genes for λ1 and the other

isotypes. Though enhancer sequences in the *J-C* introns seem to be required for transcription of rearranged genes for H and κ chains (84–88), the experimental approaches that demonstrated H and κ enhancer elements have not succeeded so far in revealing similar elements in the rearranged *λ1* gene (89).

Nonetheless, there is preliminary evidence for a λ1 enhancer. This evidence derives from gene-transfer experiments using a modified retrovirus vector (90) that contains two exogenous, inserted sequences, one a rearranged *λ1* gene and the other a Neomycin-resistance gene. The resistance gene confers on eucaryotic cells the ability to grow in what are otherwise toxic concentrations of a neomycin-like drug (G418) (91). When various cultured lines of B and T cells and fibroblasts were infected with this modified vector the lymphocytes, but not the fibroblasts, became G418-resistant, providing the resistance gene had a neighboring rearranged *λ1* gene insert (R. Cone, E. B. Reilly, in preparation). These findings suggest that a tissue-specific (i.e. a lymphocyte- or perhaps a B cell–specific) enhancer element in the *λ1* gene facilitates expression of the Neomycin-resistance gene. If further analyses support this view, similar studies with rearranged *λ2* and *λ3* genes may make it possible to determine whether or not these genes have less potent enhancer sequences than *λ1*. It should be noted, finally, that the disparity in isotype expression is evident in LPS-stimulated B cells, not in resting B cells and not, so far as is known, in the most highly differentiated B cells (plasma cells, myelomas, hybridomas). If there are significant differences among the λ-isotype enhancers, there may also be some differences between B-cell blasts and resting or fully differentiated B cells in the cellular proteins that interact with enhancer sequences.

Defective Expression of λ1 Chains in SJL Mice

λ1 chains are barely detectable in SJL mouse serum, the concentration being about 1/30th the level in other strains (72, 73). The segregation of the low serum λ1 phenotype in progeny of crosses between SJL and BALB/c, a strain with normal levels, suggests that the production of these chains is controlled by a single gene, *rλ1*, with two alleles, one ($\lambda 1^+$) associated with conventional levels, the other ($\lambda 1^{lo}$) associated with barely detectable levels (73). The *rλ1* is not linked to the major histocompatibility locus or to the Ig heavy chain locus (73). Instead, the distribution of serum λ1 values among strains of the BALB/cKe × SJL/J recombinant inbred family indicates that the defect in $\lambda 1^{lo}$ is linked to the *λ1* structural gene (92). The latter was originally identified serologically as a *λ1* allotypic marker (93), which corresponds to a recently discovered sequence polymorphism in the *Cλ1*

gene segment (94). From the intermediate serum $\lambda 1$ levels in F1 progeny of crosses between $\lambda 1$-low and $\lambda 1$-high strains it also appears that *r$\lambda 1$* acts cis to the structural $\lambda 1$ gene (73).

Though serum $\lambda 1$ concentrations are extremely low in SJL, it is possible that these mice produce some normal $\lambda 1$. Thus, adsorption of SJL serum with anti-$\lambda 1$ antibodies reduces even further the capacity of these serums to compete with authentic $\lambda 1$-Igs in radioimmunoassays (73). Moreover, when immunized with the particular dextran (alternating (1–3),(1–6) backbone) that elicits in $\lambda 1^+$ strains antibodies with a high frequency of $\lambda 1$ chains and a characteristic shared idiotype (72, 73, 95) SJL mice produce about 100-times lower levels of anti-dextran antibodies (73). However, the small amount they produce does contain $\lambda 1$ (or $\lambda 1$-like) chains and the idiotype that is characteristic of anti-dextran in $\lambda 1^+$ strains (72, 73, 95). Since, however, the V regions of $\lambda 3$ and $\lambda 1$ chains are encoded by the same V$\lambda 1$ gene segment (28), it is possible that the SJL Id$^+$ antidextran antibodies contain $\lambda 3$ instead of $\lambda 1$ chains. This possibility can now be readily evaluated (e.g. see Figures 8, 9).

When immunized with a $\lambda 1$-Ig from a $\lambda 1^+$ strain, SJL mice produce antibodies to the C domain of $\lambda 1$ chains. Indeed, an anti-C$\lambda 1$ monoclonal antibody of SJL origin is useful for detecting and measuring concentrations of $\lambda 1$ (96). The ability to make such antibodies could mean that SJL mice make no normal $\lambda 1$ chains at all and therefore respond well to immunization with them, or it could mean that the anti-C$\lambda 1$ antibodies made in SJL mice are antiallotypic, specific for the limited polymorphic difference between the C domain sequence of $\lambda 1^+$ and $\lambda 1^{lo}$ strains (see below).

The frequency of $\lambda 1$-B cells is about 100-times lower in mature SJL than in mature $\lambda 1^+$ mice (83). However, the frequency seems to be the same in newborn SJL and in newborn $\lambda 1^+$ mice (C57BL/6 and BALB/c). Evidently, with age the frequency increases in $\lambda 1^+$ strains but decreases in SJL (83). It is thus possible that the *r$\lambda 1$* gene controls not so much the number of $\lambda 1$-B cells that can be formed as the ability of these cells to be stimulated to proliferate and eventually secrete $\lambda 1$-Igs.

Recently, Arp et al (94) analyzed in DNA from SJL mice the encoding sequences for the *$\lambda 1$* gene and the nucleotides flanking these sequences, and compared the results carefully with the corresponding sequences in BALB/c ($\lambda 1^+$) DNA. The only difference found was one T-G substitution, corresponding to an alteration in the C domain protein sequence at position 155, from a Gly (GGT) in BALB/c to a Val (GTT) in SJL. This nucleotide substitution also results in a restriction enzyme (Kpn)-sensitive site in BALB/c that is lacking in SJL. The Gly/Val substitution could provide the basis for the $\lambda 1$ allotypic difference between the two strains, and

it could account also for the ability of SJL to form anti-Cλ1 antibodies in response to immunization with λ1-Ig from the C57BL/6 strain (which is a $\lambda1^+$ strain and also has the Kpn-sensitive site in its *Cλ1* gene segment).

Arp et al (94) found no sequence differences between SJL and BALB/c DNA in the heptamer and nonamer signal sequences involved in joining *V* to *J-C* gene segments, or in approximately 100 nucleotides upstream of *Vλ1*, a region that is likely to include the λ1 promoter, or in the noncoding nucleotides downstream of *Jλ1* and upstream of *Cλ1*, which contain sites that are important for RNA splicing. There are thus no grounds for supposing that the *λ1* defect in SJL arises from an ineffective *Vλ1-Jλ1* rearrangement or from abnormal initiation of transcription, or from defective RNA splicing.

It is difficult to see how a single G-T transversion, resulting in a single Gly-Val substitution in the C domain, could have so large an impact on λ1-Ig production. Nonetheless, there is an interesting correlation between this single base change and the λ1 phenotype. The BSVS mouse strain shares with SJL the λ1-low phenotype and the absence of the *Cλ1* Kpn-sensitive site (revealed by Southern blot analyses), whereas a subline of SJL developed by M. Potter has both normal λ1 levels in serum and the *Cλ1* Kpn-sensitive site of the $\lambda1^+$ strains (94).

On balance, however, it seems unlikely that the altered Cλ1 sequence in SJL and BSVS is related to the $\lambda1^{lo}$ phenotype. Indeed, both of these strains also have very low λ2 (plus λ3) levels (70), and it is possible that their low levels of λ1 reflect a more generalized regulatory defect affecting all λ gene expression. To evaluate this possibility it would be of interest to determine serum λ1 concentrations in other strains with unusually low λ2 (and λ3) serum levels, e.g. P/J, RIII/2J, C58/J, CE/J (70). It might also be useful to determine whether these other λ2 (λ3)-low strains have the same Kpn-resistant *Cλ1* site of SJL and BSVS mice. The retrovirus vector that is being used to study expression of λ1 genes [see above and (90)] may eventually prove valuable in determining the basis for defective λ1 expression in SJL and BSVS mice.

EXPANSION AND CONTRACTION OF λ GENES

Cλ Gene Segments in Inbred Mouse Strains

Sequence homologies are pronounced between the *Cλ2* and *Cλ3* gene segments and between the *Cλ1* and the unexpressed *Cλ4* segments (23–26). Likewise, the *J-C* introns of λ2 and λ3 are similar, and those of λ1 and λ4 are similar (25, 97). These relationships suggest that the four segments may have resulted from a relatively recent duplication in evolution of an ancestral

segment that contained two closely linked sequences, Cλx—Cλy, with Cλx the precursor of Cλ2, and Cλ3 and Cλy the precursors of Cλ1 and Cλ4 (25).

λ Gene Segments in Wild Mice

Another suggestion that the λ gene locus has undergone relatively rapid changes during evolution has emerged from interesting studies of wild mouse populations. Fragments of DNA from mice derived from such populations cross-hybridize strongly with probes for *Cλ* gene segments of the BALB/c mice (98, 99). This finding alone suggests pronounced sequence homology between *Cλ* sequences of inbred and feral mice. It also justifies the use of the BALB/c Cλ probes to estimate, by Southern blot hybridization assays, the number of *Cλ* gene segments in wild mouse populations. This number apparently varies from six to twelve in mice derived from two wild populations, *Mus musculus musculus* and *M. musculus domesticum* (98, 99). If inbred mice are descended from *M. musculus domesticum* (100, 101), several *Cλ* gene segments seem to have been deleted during the course of inbreeding. However, several distantly related species of the subgenus *Mus* appear to have only four *Cλ* genes, as in the inbred strains; if the inbred strains are descended from these other species then no change in the number of *Cλ* gene segments during inbreeding need be postulated. All of these considerations are, however, highly provisional, since they are based on the assumption that each positive band in a Southern blot represents a single gene segment. However, a single band could contain multiple segments and a single gene segment can give rise to more than one band.

Vλ gene segments. Southern blot analyses with probes for *Vλ* segments of BALB/c mice show no differences among the inbred mouse strains that have been examined. All appear to have two *Vλ* gene segments on restriction fragments that do not differ in size among the strains [(23) B. Blomberg, personal communication]. However, there appear to be at least three *Vλ* segments in *M. musculus domesticus* (102), raising the possibility again that a loss of λ gene segments (in this case *V*) occurred during the inbreeding process.

It is possible that many repetitive sequences or an unusual amount of sequence homology is present in the chromosome that contains mouse λ genes [chromosome 16 (29)]. Such sequences could provide a large target for recombination between sister chromatids and increase opportunities for amplification and contraction in the number of *Cλ* and of *Vλ* gene segments. A rough correlation between the number of *Vλ* and *Cλ* gene segments in *Mus* generally supports the notion that the recombination unit includes a single *Vλ* and linked *Jλ-Cλ* gene segments, as in the BALB/c mouse (23–26, 97) (see Figure 4). However, the evidence for inclusion of a *Vλ*

segment in this unit is still tenuous, because the $V\lambda$ has not yet been physically linked to the $C\lambda$ gene segments.

$C\lambda$ Gene Segments in Humans

Serological studies and amino acid sequences of constant region peptides from human Bence-Jones proteins led to the first suggestion that there are multiple nonallelic forms of λ chains (103–107). Detailed amino acid sequences indicated that there are at least four nonallelic forms (i.e. isotypes) in humans, but the exact number has not been clear because of difficulty in distinguishing allelic from nonallelic variants in outbred populations. Recent studies of human DNA initiated with λ cDNA probes of mouse (BALB/c) origin, have led to the isolation of three $C\lambda$ gene segments that correspond to known human λ isotypes (108); three additional $C\lambda$ gene segments have been identified but not yet sequenced. All six reside in tandem in a 50 kb segment on chromosome 22 (108). As yet unlinked to this cluster are three additional λ-like segments and several DNA fragments that cross-hybridize weakly with human and mouse λ probes. How many of these genes are functional is not clear, but an unusual λ pseudogene, identified on a different chromosome, has the sequence expected of cDNA for a processed RNA molecule, i.e. one from which the noncoding sequence between the *J* and *C* gene segments has been excised (109).

Aside from the multiplicity of $C\lambda$ gene segments, the extent to which the organization of human and mouse λ gene segments are alike is still not clear. It is not known, for instance, if each human $C\lambda$ gene segment is associated with its own $J\lambda$ gene segment, or if the $J\lambda$ segments in the human genome are clustered (as in the *H* and *K* gene families). The number of $V\lambda$ gene segments is also unknown, but it is expected to be substantially greater in man than in inbred mice, because the proportion of human Igs with λ chains are almost 10-times greater than mouse Igs with these chains (about 40% vs. about 5%).

CONCLUDING REMARKS

This review emphasizes three properties of λ chains and genes in inbred mice: their organization and diversity, the relationships between their primary structure and ligand-binding activity, and the different frequencies with which the several isotypic forms are found in serum Ig, spleen B cells, and antibodies to various haptenic groups. The simplicity of this Ig gene family has made it possible to attempt a quantitative assessment of the extent to which various mechanisms contribute Ig V region sequence diversity. For λ1 chains, somatic mutation is clearly the great diversifier:

V/J junctional amino acid substitutions, arising from imprecision in gene segment assembly, are responsible for structural variations that are few in number but large in impact on ligand-binding activity: However, V-J combinatorial variation due to recombination of different V and J gene segments is virtually nonexistent, at least when the individual isotypes are considered.

Several provocative regulatory problems have emerged from studies of these chains. One is the much greater frequency of $\lambda 1$ than $\lambda 2$ or $\lambda 3$ chains. The difference between $\lambda 1$ and $\lambda 3$ is especially intriguing because both use the same V gene segment. Another regulatory problem of interest is the defective expression of $\lambda 1$ (and also the other λ chain isotypes) in SJL mice. Despite the simplicity of the λ-gene family a solution to these regulatory problems is not yet at hand. It is possible, nonetheless, that further analysis of these chains and genes will contribute as much to our understanding of regulation problems as they have to our understanding of the sequence organization and diversity of Ig chains and genes in general.

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CONTENTS

Ann. Rev. Immunol. 1985. 3:367–96

GENETICS AND EXPRESSION OF MOUSE Ia ANTIGENS

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INTRODUCTION

The application of both monoclonal antibody technology and the techniques of recombinant DNA to problems in immunology has been a driving force in the recent rapid increase in our understanding of the functions and molecular nature of the mouse H-2–linked immune response (Ir) genes. Immune response genes in mice were originally defined as the genetic elements that control the differential ability of various inbred mouse strains to respond to certain antigens with a limited range of antigenic determinants (1–3); the genes were mapped using inbred congenic mouse strains to the I region of the mouse major histocompatibility complex (MHC) on chromosome 17 between H-2K and H-2S (4, 5). That the inability of a particular strain to respond to a given antigen was due to a helper–T cell defect suggested initially that the products of the Ir genes were foreign antigen-specific T-cell receptors encoded within the I region (4). At the time of their discovery, the products of the Ir genes were not clearly identified with the serologically defined Ia antigens (6, 7), which also map within the I region. However, the accumulation of a large body of evidence over the past decade includes a firmer understanding of the cellular interactions and molecular recognition processes involved in the generation of specific immune responses; the Ia antigens are implicated as the structural genes for Ir gene–controlled functions. Most immunologists now agree that Ia antigens are the products of Ir genes.

Ia antigens are recognition structures, expressed on B lymphocytes and on antigen-presenting macrophages, that mediate a wide variety of immunological phenomena, including the activation of lymphocytes in mixed lymphocyte culture (MLC) (8) and the generation of cytotoxic

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lymphocytes (9), as well as the phenomenon known as MHC restriction. Interaction of regulatory T cells with macrophages and other lymphocytes is I-region restricted; Ia antigens serve as the restricting elements by defining the context in which T cells "see" foreign antigen on the surfaces of Ia-expressing cells (10–12). Precursor T cells are primed by a particular combination of self Ia and foreign antigen, and their progeny are able to proliferate only in response to the same antigen in combination with the Ia molecule of the same genetic type (haplotype) (13). Therefore, T-cell activation generally requires that antigen-presenting cells and T-cell populations be from mice of the same haplotype in the I region (14, 15).

The close functional association between MHC restriction and Ir gene control was elucidated through experiments first using anti-Ia antisera (16–18), and later and more convincingly, anti-Ia monoclonal antibodies (19) to block the T cell–proliferative response of responder strains to antigens known to be under Ir-gene control in the in vitro T cell–proliferation assay (20, 21). In addition, experiments showed that the response to antigens such as the copolymer poly(GluLysTyr), or (GLT), which are controlled by two complementing Ir-gene loci can be blocked by anti-Ia monoclonal antibodies directed against an Ia-antigen complex composed of subunits encoded in the same two genetic regions (19). Together these experiments provided convincing evidence that Ia antigens mediate Ir-gene functions. Indeed, Ia antigens have been shown to be expressed on cells that mediate Ir-gene functions and on many of the soluble factors important in regulating immune responsiveness (22); no recombinations have been identified that separate Ia from Ir loci within the same I subregion.

It is still not clear at the molecular level exactly how Ia antigens control responsiveness to antigens under Ir-gene control. Evidence suggests that Ir genes are able to discriminate subtle differences in the structures of antigens such as insulin (23), lysozyme (24), and cytochrome c (25), and that Ir genes somehow select antigenic determinants for presentation to T cells (23). One of the obvious concerns of immunologists has been to explain the ability of Ir genes to control responsiveness to such a wide variety of simple and complex antigens through the few structural Ia products so far characterized. Although some non-H-2-linked Ir genes have been identified (26–28), most Ir genes do map within the I region. In the past several years the availability of I region locus-specific molecular probes has facilitated dissection of the I region at the molecular level. Together with the recent application of techniques of DNA-mediated gene transfer to class II genes, these findings have begun to allow the correlation of primary structure and serology of I-region products with their functions as genetic restrictors of the immune response, and thus they have cleared the way to a more

complete understanding of the role played by Ia antigens in the regulation of I region–linked immune responses.

GENETIC ORGANIZATION OF THE I REGION

The I region has been divided into five subregions by serological analysis of recombinant H-2 haplotypes; these are: I-A, I-B, I-J, I-E, and I-C (29). The crossover positions in H-2 recombinant strains define the subregion boundaries. Only four I region–associated (Ia) products have been identified by both serological and biochemical analysis (30, 31). The I-A subregion contains at least three loci that encode serologically detectable class II polypeptide products: A_β, A_α, and E_β (32). The I-E subregion contains a fourth class II locus that encodes the E_α polypeptide (32). No protein products have been found that map to the I-B subregion, originally defined in two H-2 recombinant strains to control responsiveness to several antigens, including LDH_B (33) and mouse IgG_{2a} myeloma protein (34). It has been suggested that the I-B subregion is a genetic artefact, and there is evidence that responses mapping to I-B are in fact jointly controlled by products encoded by the I-A and I-E subregion (35). The I-J subregion, defined serologically, has been thought to encode determinants present on suppressor T cells and soluble factors involved in the regulation of suppressor functions, but so far no distinct I-J locus has been found (see I-J: THE ELUSIVE GENE). The I-C subregion may control the generation of suppressor T cells in the mixed leucocyte reaction (MLR) (36) and play a role in the functioning of immune suppression (Is) genes (37). I-C-encoded serologic determinants have been found on MLR suppressor factors, but these have not been biochemically characterized.

Southern blot analysis of mouse genomic DNA with class II molecular probes has suggested that class II genes exist in single-copy in the genome and that there are no more than two α genes and six β genes encoded in the mouse genome (38, 39), in contrast to the human genome which may encode as many as six functional α genes and seven β genes (40). Chromosomal walking through the I region by the ordering of overlapping cosmid clones (39) as well as genetic mapping by restriction enzyme-fragment polymorphism (41, 42) have allowed chromosomal mapping of the loci encoding the four defined class II products in the mouse, as well as identification of additional I region–associated loci that encode sequences homologous to previously defined class II genes of mouse and man (Figure 1). A second class II β gene, $E_{\beta 2}$, was identified by cross-hybridization to the human DC_β gene and mapped between the E_α and E_β loci (39). The 3′ exons of this gene are E_β-like by hybridization (39), while the 5′ exons show no cross-

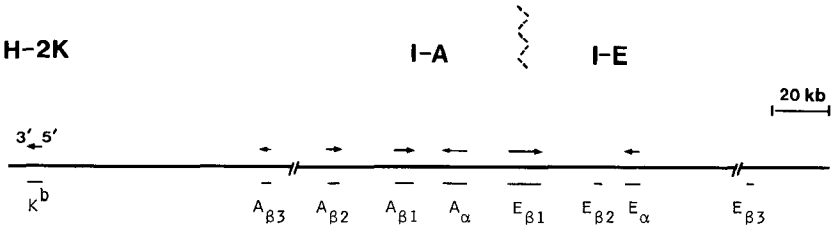

Figure 1 Organization of class-II MHC genes. 5′ and 3′ refer to the direction of transcription. (Reproduced with permission from 38.)

hybridization to an E_β probe but instead hybridize to a human DR_β probe (N. Braunstein, R. Germain, personal communication). It is not known whether this gene is transcribed, but Northern blot analysis of mouse RNA with an E^k_β cDNA probe shows only one band, at 1.3 kilobase (kb), the size of the mature E_β mRNA (L. Mengle-Gaw, unpublished results). Thus, if this second gene is transcribed, its transcript is either the same size as E_β or transcribed at too low a frequency to be detected by Northern analysis. Recently, a second A_β-like sequence has been identified—$A_{\beta 2}$—that maps 20 kb centromeric to the A_β gene and encodes an exon homologous to the second external-protein domains of class II β chains (43). Additional flanking exons have been identified by DNA sequencing, but these show lower homology to characterized class-II genes (D. Larhammer, personal communication). Additional A_β- and E_β-homologous sequences—$A_{\beta 3}$ and $E_{\beta 3}$—have been identified by chromosomal walking; $A_{\beta 3}$ maps 75 kb telomeric of K^b, while $E_{\beta 3}$ maps to either the I-C or the S subregion by analysis of restriction-fragment polymorphisms in intra–I region recombinant strains (G. Widera, C. Wake, R. Flavell, personal communication) (Figure 1). There is evidence that $A_{\beta 2}$ is transcriptionally active in spleen (D. Larhammer, personal communication), but whether any of the other additional sequences are functional (i.e. transcribed and translated into functional protein products) is unknown at present.

STRUCTURE AND EXPRESSION OF Ia ANTIGENS

Ia antigens are highly polymorphic, class II MHC, cell-surface glycoprotein molecules expressed predominantly on B lymphocytes and antigen-presenting macrophages (23, 44, 45). Secreted, soluble factors involved in the regulation of immune responsiveness (46–49) have also been shown to carry class II antigenic determinants, and human and mouse class II molecules are expressed on a large fraction of concanavalin A (Con A)–activated T cells (50, 51).

Structure of Ia Antigen Complexes

Initial serological and biochemical characterization of the Ia antigens defined the nature of the antigenic components and their overall protein structure. Immunoprecipitation by anti-Ia antisera and two-dimensional gel electrophoresis showed two heterodimeric molecular complexes, I-A and I-E, each composed of two protein subunits, a heavy α chain (M_r = 34,000) and a light β chain (M_r = 28,000) that associate noncovalently in the cell membrane (30). The two subunits of the I-A complex ($A_\alpha A_\beta$) are separately encoded within the I-A subregion (32), as is the light chain (E_β) of the I-E complex (32); E_α is encoded within the I-E subregion (32). The association of polypeptide chains encoded in both the I-A and I-E subregions to form the I-E complex defines the genetic basis for the phenomenon of Ir gene complementation (32). It has been shown that heterozygous F1 hybrid strains express variable levels of both parental and transcomplementing (hybrid) Ia antigens, so that complementation between two I region loci can occur in the *cis*- or *trans*-chromosomal position (32, 52, 53). Furthermore, at least some of these transcomplementing Ia molecules in F1 mice react with anti-Ia antisera and are functional—that is, able to restrict antigen recognition and to serve as allorecognition elements in the stimulation of MLR responses by cloned T cells (52). The expression of homodimeric (i.e. $A_\alpha A_\alpha$) complexes and I-A/I-E hybrids (i.e. $A_\alpha E_\beta$ or $A_\alpha E_\alpha$) has not been shown, but the possibility that they may exist at levels too low to detect by immunoprecipitation cannot be ruled out.

On the basis of sequence homologies at the protein and nucleotide levels, the analog in the human MHC (HLA) of the I-A complex appears to be DC (54), while the human analog of the I-E molecule is presumed to be DR (55). Both human class II complexes are also composed of two protein subunits, a heavy chain and a light chain, that show structural and functional homology to their mouse Ia analogs (56).

Role of the Invariant Chain in Ia Expression

The α and β subunits of the Ia antigens are associated intracellularly with a third glycoprotein, the invariant chain, I_i, so called because it shows little allelic protein polymorphism among different strains of mice (57). I_i is a basic polypeptide with a M_r of 31,000 daltons that is coprecipitated with Ia α and β chains in immunoprecipitations using anti-Ia antisera or monoclonal antibodies (57). Two-dimensional gel electrophoresis and tryptic peptide mapping have shown that I_i is methionine-rich and contains two asparagine-linked carbohydrate side chains (58–60). The exact role played by I_i in the functioning of Ia molecules is unclear. I_i associates noncovalently with Ia α and β chains in the membrane of the endoplasmic

reticulum, but has not been detected in association with cell-surface Ia (58, 61); this suggests a role for the protein in intracellular transport or assembly of Ia subunits on the cell surface (57, 58, 62). It has been shown that I_i and Ia can be coinduced in some macrophage cell lines with γ-interferon (63); although the synthesis of Ia α, β, and I_i chains is coordinately regulated, I_i is not linked to the H-2 complex (64), nor is the human invariant chain, DR_γ, linked to the human MHC human leucocyte antigen (HLA) [65, H. Erlich, personal communication].

Class-II α and β Polypeptides Are Folded into Distinct Domains

Biochemical analysis of class II products has revealed their basic protein structure, but protein-sequence analysis of the Ia antigens has been limited by the difficulty in purifying adequate quantities from the amounts present in and on lymphocytes and macrophages. Papain cleavage of native HLA-DR antigens has shown that molecule to be composed of a large extracellular domain, a short membrane-spanning region, and an intracytoplasmic tail (66). Limited proteolysis of human and mouse class II light chains using chymotrypsin and trypsin shows the extracellular region to consist of two distinct domains (67), an observation since extended by sequence comparisons to the human and mouse class II heavy chains.

The recent advances in molecular cloning techniques have allowed isolation and characterization of many of the human and mouse class II genes. Genes for multiple alleles of all of the known mouse Ia polypeptides have been isolated over the past two years and their characterization together with the earlier serological and biochemical data has allowed a detailed understanding of the structure of the Ia molecular complexes (Figure 2).

Both the α and β polypeptide chains are composed of five functional protein domains (41, 43, 67–70): (*a*) a hydrophobic leader peptide of approximately 25 amino acids, whose function is to guide and insert the nascent polypeptide into the membrane, after which it is then enzymatically removed and therefore is absent from the mature cell-surface form of the protein; (*b*) an N-terminal (membrane-distal) extracellular domain (α_1, β_1) of 84–88 amino acid residues in the Ia α chains and 96 residues in the β chains, of variable chemical character (see ALLELIC POLYMORPHISM IN THE Ia α AND β GENES); (*c*) a second (membrane-proximal) extracellular domain (α_2, β_2) of approximately 95 residues that shows significant protein-sequence homology to immunoglobulin–constant region domains, β_2-microglobulin, and the membrane-proximal domain (α_3) of human class I heavy chains (71, 72); (*d*) a hydrophobic transmembrane region of 23–25 residues that terminates with a cluster of

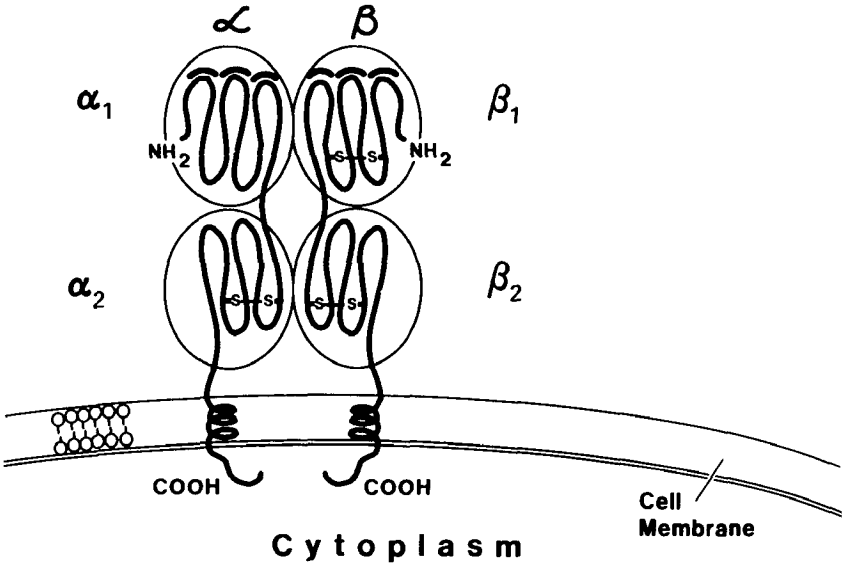

Figure 2 Schematic representation of the structure and membrane orientation of Ia molecules. Solid overlining represents possible tertiary orientation of Ia hypervariable regions.

positively charged residues on the cytoplasmic side of the membrane, thought to serve as an anchor for the protein in the membrane; and (*e*) a cytoplasmic tail region containing a high proportion of charged and hydrophilic residues. There is also a short (11–13 residues) protease-sensitive connecting peptide, glutamic acid and proline-rich in the α chains and serine-rich in the β chains, that links the second external domain to the transmembrane region. The class II β chains are encoded by six exons, one exon corresponding to each of the five major protein domains, and an additional exon encoding the 3′ untranslated region (41, 69). The structure of the class II α genes is similar, except that the transmembrane and cytoplasmic tail regions are encoded by a single exon, so that the genes are comprised of five exons (41, 73).

Glycosylation of Class-II Polypeptides

The murine class-II α chains contain two potential glycosylation sites—one in the N-terminal domain at residue 78 and one in the second external domain at residue 118. The β chains have only one potential glycosylation site, at residue 19 or 20 in the N-terminal domain. The function of carbohydrate side chains on class-II molecules is unclear. Glycosylation does not appear to be required for chain association or expression of class-II molecules on the cell surface (74). Alloantigenicity of these molecules also

does not appear to be affected by the presence or absence of N-linked glycans, as class II products react with specific alloantisera after tunicamycin treatment (75). Whether they play some role in the modulation of cellular interactions is not known.

Cysteine Bridges Form Intrachain Disulfide Loops

Both external domains of the class-II β chains contain cysteine disulfide bridges: A loop of 64 amino acids is formed by a bridge between cysteine residues at positions 15 or 16 and at 79 or 80 in the N-terminal domain, and cysteines at positions 118 and 174 form a disulfide loop of 56 amino acids in the second external domain. The class-II α chains contain only one disulfide loop, of 56 amino acids, in the second external domain between cysteines at positions 107 and 163. On the basis of biochemical evidence (66, 76) and protein-sequence homology to immunoglobulin domains (71, 72), it appears that the $\alpha 2$ and $\beta 2$ domains of class II polypeptides exhibit the immunoglobulin fold, a tertiary folding of alternating β-pleated sheets and bends (77, 78). The secondary and tertiary structures of the other class II domains are unknown, although there is some evidence that $\alpha 1$ and $\beta 1$ domains alternate β-pleated sheets and bends as well [(79); C. Benoist, unpublished results], and that the transmembrane regions of these molecules are folded into α-helixes (78).

ALLELIC POLYMORPHISM IN THE Ia α AND β GENES

It is not known at the molecular level exactly how the two Ia complexes mediate Ir gene functions, especially MHC restriction of specific antibody responses. Elucidation of the molecular basis for the functioning of Ir genes is eagerly sought, and will, it is hoped, illuminate general mechanisms for the molecular modulation of cellular interactions, cell triggering, and differentiation. The phenomenon of I-region restriction certainly implies that mice of different haplotypes show functionally significant protein-sequence polymorphism in their I region–encoded protein products. Biochemical analysis of the polypeptide chains comprising the Ia molecular complexes, by two-dimensional gel electrophoresis, tryptic peptide mapping, and amino-terminal protein sequence determination, have generally shown significant protein polymorphism among alleles of the β chains, while the α chains were long thought to be relatively nonpolymorphic (80, 81, 82). Several biochemical studies, however, suggested that the A_α chain shows a high level of allelic variability (83, 84). The recent isolation of genomic and cDNA clones for multiple alleles of Ia genes and comparison

of their nucleotide and predicted protein sequences have confirmed this earlier data for E_α but revealed that A_α is significantly more polymorphic than predicted from earlier biochemical analysis.

Comparison of Allelic Sequences of Cloned Ia Genes

Allelic forms of the E_α gene have been cloned from two mouse haplotypes—k and d (41, 73). The A_α gene has been cloned from six mouse haplotypes: k, d, b, u, f, and q (85, 86). Four alleles of E_β have been cloned—k, d, b, and u (69, 79, 87, 88); and A_β alleles from k-, d-, and b-haplotype mice have been cloned (43, 70). Comparison of nucleotide sequences of allelic forms of each Ia gene shows that allelic nucleotide variability is extensive and clustered in the region encoding the first external protein domain for A_α, A_β, and E_β; comparison of the two E_α alleles shows very few differences in nucleotide sequence. The ratio of productive (i.e. leading to amino acid substitution) to silent (i.e. maintaining the same amino acid residue) nucleotide changes for each protein domain of the α and β chains is calculated and shown in Tables 1 and 2. A larger ratio generally indicates a relatively lower degree of sequence constraint. The values show tremendous selection for productive change in $\alpha 1$ and $\beta 1$ domains, while there is strong selective pressure for sequence constraint over the remainder of the proteins.

Comparison of predicted protein sequences for A_α, A_β, and E_β alleles shows that allelic amino acid variability is clustered in three or four short stretches of protein sequence within the first domain (Tables 1 and 2), separated by stretches of protein sequence conserved among alleles at a particular locus as well as between α or β loci (79, 87). Comparison of six A_α alleles shows that 70% of the total amino acid substitutions cluster in three stretches of protein sequence comprising 12% of the total protein. For A_β, 8.4% of the total protein sequence contains 60% of the variability. For E_β, 84% of allelic amino acid substitutions occur in 17% of the total protein sequence. Furthermore, most of the allelic amino acid substitutions in the variable regions in these three genes are chemically nonconservative and therefore would be expected to affect local secondary structure in these first domain clusters (79, 86). E_α alleles only show 1% average protein-sequence polymorphism and contain about as many conservative as nonconservative changes scattered throughout the protein sequence. It has been observed (86) that certain alleles show more protein-sequence homology with one another than with other alleles. The b, u, and k alleles of A_α are most similar to each other and show much variability when compared with the other alleles; the f and q alleles also show sequence similarity; and the d allele seems most different, which may indicate that it diverged from the other alleles relatively long ago. Comparison of the E_β alleles shows the k

Table 1 Amino acid differences between pairs of Ia α chain alleles within specific polypeptide regions of the mature proteins

	Allelic pair	Total protein	D1	D2	TM-C	A_α hypervariable regions 10–15	44–49	53–59	69–77
A_α	k,d	14	12	2	0	2	2	3	3
	k,b	11	10	1	0	2	0	4	4
	k,f	13	11	2	0	3	1	4	1
	k,u	7[a]	6[a]	1	0	1	1	2	2
	k,q	15[b]	11[b]	3	1	1[b]	0	4	3
	d,b	16	13	3	0	3	2	3	3
	d,f	11	11	0	0	2	1	3	2
	d,u	18[a]	15[a]	3	0	3	3	4	3
	d,q	14[b]	12[b]	1	1	2[b]	2	3	4
	b,f	12	9	3	0	1	1	1	3
	b,u	7[a]	7[a]	0	0	2	1	2	2
	b,q	14[b]	9[b]	4	1	1[b]	0	1	4
	f,u	13[a]	10[a]	3	0	2	2	2	1
	f,q	8[b]	6[b]	1	1	1[b]	1	0	2
	u,q	13[b]	8[b]	4	1	0[b]	1	2	2
Average percentage allelic amino acid variability		5.4	12.1	2.2	0.65	34.7	20.0	36.2	28.9
Total productive: silent nucleotide substitutions[c]		59:14	9:26	1:7	10:0	4:1	19:9	15:0	
E_α	k,d	3	0	2	1				
Average percentage allelic amino acid variability		1.3	0	2.2	1.9				
Total ratio productive to silent nucleotide substitutions[c]			0:1	2:4	1:0				

[a] Residues 1–5 of A_α^u not available for comparison.
[b] Residues 1–12 of A_α^q not available for comparison.
[c] Sum of independently-scored allelic substitutions from the A_α^k or E_α^k sequence.

Table 2 Amino acid differences between pairs of Ia β chain alleles within specific polypeptide regions of the mature proteins

	Allelic pair	Total protein[a]	D1[a]	D2	TM-C	A_β hypervariable regions 9–17	63–68	84–89
A_β	d,k	20	16	4	0	5	4	3
	d,b	12	8	4	0	3	0	1
	k,b	17	15	2	0	4	4	3
Average percentage allelic amino acid variability		6.4	12.8	3.2	0	44.4	44.4	38.9
Total productive: silent nucleotide substitutions[b]			33:4	9:17	0:3	13:0	4:0	7:0

	Allelic pair	Total protein	D1	D2	TM-C	E_β hypervariable regions 1–13	27–39	68–75	87–93
E_β	k,u	22[c]	21[c]	1	0	7[c]	5	3	3
	k,b	5[c]	5[c]	0	0	1[c]	1	0	3
	k,d	22[c]	21[c]	1	0	6[c]	5	4	3
	u,b	19	18	1	0	7	5	3	0
	u,d	16	15	1	0	5	5	3	0
	b,d	17	16	1	0	5	4	4	0
Average percentage allelic amino acid variability		7.1	16.8	0.81	0	41.2	32.1	35.4	21.4
Total ratio productive to silent nucleotide substitutions[d]			65:8	2:4	0:4	23.1	13:5	9:0	12:0

[a] Residues 1–5 not available for comparison.
[b] Sum of independently-scored allelic substitutions from the A^d_β sequence.
[c] Residue 1 of E^k_β not available for comparison.
[d] Sum of independently-scored allelic substitutions from the E^k_β sequence.

and b alleles to be most similar, and both to be quite different from the other alleles. The A_β alleles, however, all exhibit the same degree of variation when compared pairwise.

Interlocus Class-II Protein Homologies

Comparison of A_α vs E_α and A_β vs E_β protein sequences may give some clues to the functions of individual protein domains. The α chains show 50% overall sequence homology, with 78% sequence conservation in the transmembrane regions (85). It has been suggested that α chains might associate with β chains or I_i through this highly conserved region (85). The α chains show least homology in their cytoplasmic tails, an observation which suggests that the proteins may interact with different molecules in the cytoplasm (85). The presence of two serine residues that may serve as sites for phosphorylation in A_α, and their corresponding absence in E_α is interesting in this regard (85). Ia β chains exhibit 65% overall protein-sequence homology (87). Again, the transmembrane regions show the most homology, while the cytoplasmic tails are most divergent (87). The E_β alleles contain two conserved serine residues in this region, while the A_β alleles contain one such residue (87). The second domains of both the α and β chains show strong sequence homology among alleles at each locus, as well as between α or β loci. Eighty-six percent of the residues conserved in immunoglobulin domains and thought to direct protein folding into an Ig-like domain are also conserved in the second domains of all alleles of both β chains, and 68% of the residues are conserved among all alleles of both α chains, supporting the notion that the second domains of class-II chains are folded into Ig-like domains (79).

A Model Correlating Ia Primary Structure with Functional Polymorphism

It is tempting to speculate that the clustered nature of allelic polymorphism in the first domains of A_α, A_β, and E_β implicates these regions as recognition sites for interaction with foreign antigens or a T-cell receptor. Although the physical interaction of Ia molecules with foreign antigen has not been proven, this clustering phenomenon and the nature of the amino acid substitutions within these regions supports this intriguing possibility, and the presence of multiple antigen- or receptor-binding sites on each Ia chain has been suggested as one way to increase the site diversity of the limited number of Ia loci apparently expressed in the mouse (89). We have suggested that Ia chains may fold to allow juxtaposition of all three or four variable regions at the surface of the folded molecule where they could interact with antigen or T-cell receptor (87) (Figure 2). In fact, the lengths and spacings of the clustered Ia variable regions are reminiscent of

immunoglobulin hypervariable regions, and these Ia hypervariable regions may likewise fold to form an active site for antigen binding (86, 87). Alternatively, Ia hypervariable regions may fold to form several separate binding sites on the molecule. Without knowledge of the native three-dimensional structure of Ia molecules, this obviously remains speculative.

CORRELATION OF Ia ANTIGEN PRIMARY STRUCTURE WITH SEROLOGICAL AND FUNCTIONAL DATA

Antigen-Specific Immune Responses Controlled Through a Specific Ia Molecule

Ir gene control of immune responsiveness to antigens under the control of a single Ir gene has been mapped serologically to the I-A subregion, to the $A_\alpha A_\beta$ molecule, while response to those antigens under dual Ir gene control maps to both the I-A and the I-E subregions, since responses to these antigens are mediated by the $E_\alpha E_\beta$ molecule (90). Although responses to many antigens have been mapped serologically to either the I-A or I-E molecule, the control of relatively few responses has been mapped to a particular chain of an Ia complex, if indeed a single chain is ever responsible for antigen presentation. It is particularly difficult in the case of the I-A molecule, owing to the lack of genetic recombinants separating the A_α and A_β loci within the I-A subregion and the structural complexity of both I-A protein subunits, which make it difficult to attribute response patterns to allelic polymorphisms in a single chain. Owing to the extreme allelic polymorphism in both chains, it is also more likely that both I-A chains do function directly in antigen presentation. However, Ir-gene control of the responses to several antigens controlled by the I-E molecule has been localized to the I-E_β chain. With Ia-antigen primary-sequence data available, we can begin to dissect the molecular features that distinguish responder and nonresponder phenotypes.

SPECIFIC IMMUNE RESPONSES CONTROLLED THROUGH THE E_β CHAIN The response to the synthetic polypeptide poly(GluLysPhe) or GLPhe, is controlled by the I-E molecule (91). It has been shown that histocompatibility at I-A, but not I-E, is required for antigen presentation of GLPhe to primed responder T cells (92), indicating that the structurally similar E_α chains from many haplotypes are functionally equivalent and that response to GLPhe is probably controlled by I-E_β. Similarly, the responses to the synthetic copolymer poly(GluLysTyr) or GLT (19, 90), and to pigeon cytochrome *c* (93, 94) are probably controlled by I-E_β. Examination of the protein sequences of the four available E_β alleles shows that responder or

nonresponder status to these three antigens can in fact be correlated with certain distinctive molecular features of the E_β polypeptide chain. The E_β protein-sequence differences between responder and nonresponder alleles for all three antigens cluster in E_β hypervariable regions. While the response patterns to these antigens are consistent with the idea that these responses are mediated through hypervariable regions in I-E_β, it is not clear that responsiveness is determined by the presence or absence of a specific hypervariable region polypeptide sequence in E_β. Since the E_β chain appears to be expressed on the cell surface only in association with an E_α chain, and since different E_α alleles seem to be functionally equivalent by apparent virtue of their very similar sequences, it is difficult to determine whether E_β restriction sites can exist independent of sequence contribution from the E_α chain. Certainly though, since the α chains of responders and nonresponders are so similar, I-E mediated responsiveness must be determined by allelic polymorphic regions in the E_β chain. While a single hypervariable region sequence may confer responsiveness to a certain antigen, it is more likely that the overall chemical character of the site determines how the I-E molecule will present a particular antigen to T cells. If the I-E molecule folds so that all four E_β hypervariable regions form a single binding site, the additive affinity of the binding site for antigen would likely determine responsiveness. Without knowledge of the tertiary structure of Ia molecules, it is nearly impossible to attribute responsiveness to a given antigen with the presence of a certain responder Ia-polypeptide sequence.

Functional Significance of Ia Hypervariable Regions

Perhaps the strongest argument for the direct functional involvement of Ia hypervariable regions in T-cell stimulation is the isolation of an alloreactive T-cell clone that was shown by monoclonal antibody blocking and by Ia nucleotide and protein-sequence comparison, to recognize a determinant spanning one of the E_β hypervariable regions, residues 68–75 (95). Other data implicates the same determinant in presentation of sheep insulin to T cells. Substitution of three amino acid residues obliterates its ability to present sheep insulin, but instead allows bovine insulin presentation (96) (Table 3).

The alloreactive T-cell clone that recognizes residues 68–75 of the E_β polypeptide chain was shown to be stimulated by both $E_\alpha^k E_\beta^b$ and $A_\alpha^b A_\beta^{bm12}$ molecules on stimulator cells, but not by the $A_\alpha^b A_\beta^b$ molecule (95). The differential stimulation of a T-cell clone by Ia molecules that have subtle but defined sequence differences suggests that responsiveness is determined by key sequences on Ia molecules. Similarly, diseases shown to be strongly associated with particular H-2 or HLA alleles may be more accurately

Table 3 Multiple Ir gene functions controlled by a single defined Ia peptide region

Residues 68–75 of the E^b_β polypeptide control:		Reference
Allogeneic T-cell recognition		95, 96
Antigen presentation		96
Controls response to	Does not control response to	
beef insulin	(Phe,G)-A--L	
sheep insulin	(T,G)-A--L	
	(H,G)-A--L	
E_β Allelic hypervariable region		79
Donor sequence in gene conversion event generating the BM12 mutation		88, 95

attributable to a particular peptide sequence present differentially on disease-associated or normal alleles. This suggests a role for Ia sequence-specific DNA probes in the diagnosis and etiology of MHC-associated diseases.

GENERATION OF POLYMORPHISM IN CLASS II GENES

Gene Conversion Between Class-II β Genes

Comparison of the sequences of genomic clones for the d and b alleles of E_β shows a higher nucleotide substitution rate in exons than in the noncoding introns (88); this suggests that accumulation of allelic variability is somehow selected for in protein-coding regions. Presumably, fixation of random mutations by phenotypic selection is an important mechanism for generating diversity in Ia molecules. I-region restriction of immune responsiveness most likely depends upon allelic Ia protein polymorphism, which certainly expands the ability of the population to respond to a wider variety of foreign antigens. In addition, allelic polymorphism that has clusters of highly polymorphic sequence interspersed with regions conserved both between β loci and among alleles at each β locus is a pattern that suggests that polymorphism in β chains might be generated in part by micro–gene conversion events—the intergenic transfer of a block of nucleotide sequence from one donor to another recipient gene, mediated by flanking regions of interlocus homology, through the resolution of heteroduplex hybrid DNA formed as a meiotic or mitotic recombination intermediate (97).

Gene conversion has been proposed as a mechanism to generate

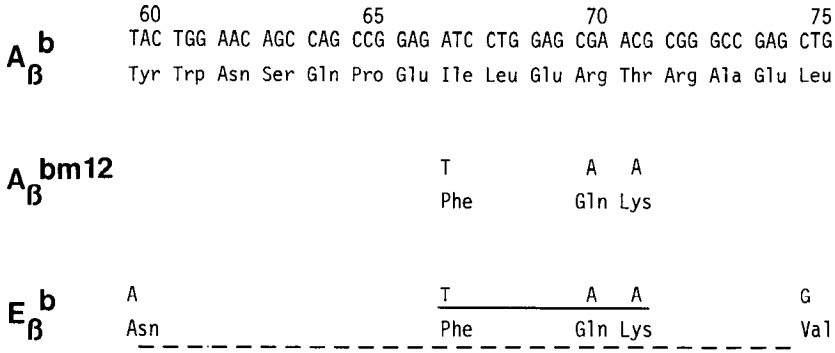

Figure 3 Comparison of DNA and amino acid sequences of the A^b_β gene, the mutant A^b_β gene of the B6.C-H-2 (bm12) mouse, and the E^b_β donor gene in the first domain region of the bm12 mutation. Solid line shows the predicted minimum, and the dashed line the maximum, extent of sequence transferred from the E^b_β gene in the hypothesized gene-conversion event generating the bm12 mutant. (Adapted from 95.)

polymorphism in class I genes (98–100), with the identification of a donor gene mapping to the Qa2.3 locus for the mutation event generating the K^b mutant, bm1 (98). Recently, this observation has been extended to class II genes. Comparison of the nucleotide sequences of E^b_β and the A^{bm12}_β chain from the A_β mutant strain B6.C-H-2^{bm12} (bm12) provided evidence that the bm12 mutation was generated by transfer of a stretch of 14–44 nucleotides from the exon encoding the first domain of E^b_β to the corresponding region in A^b_β (88, 95) (Figure 3, Table 3).

Long-Term Effects of Gene Conversion

Depending upon the circumstances, gene conversion may be viewed as a mechanism that potentially either generates diversity between the products of donor and recipient genes or corrects sequence differences between homologous genes and thus acts to homogenize sequences. Net effect of gene conversion on the sequences of homologous genes probably depends in large part upon the relative rates of conversion vs random mutation (101–103). Conversion events may generate polymorphism between genes whose rate of random mutation is greater than the conversion rate between them; conversely, when the random mutation rate is much lower than the rate at which sequences are transferred between genes, the net effect of conversion may be to homogenize sequences. Furthermore, directionality in the conversion process is likely to be a major factor in determining its net effect: Unidirectional gene conversion is presumably necessary for the maintenance of sequence polymorphism over time (103). Gene conversion events may have different outcomes in different parts of class II β genes. In

exons encoding the first domains, where the rate of random mutation fixation is high due to phenotypic selection, the relative rate of gene conversion may be low, and its net effect to generate polymorphism. In introns and other exons where there appears to be much less selection for fixation of changes and the rate of accumulation of random mutations is lower, the relative rate of gene conversion may be higher, and the net effect of conversion in these regions is therefore to correct sequence differences and maintain sequence homology. Gene conversion as a mechanism which maintains sequence homogeneity has been suggested for the globin (104, 105) and immunoglobulin (106) gene families.

Although A_α genes exhibit similar clustering of extensive allelic polymorphism, in contrast to the class II β genes there is no evidence that gene conversion events play a role in determining the patterns of allelic polymorphism in A_α alleles (86), as suitable donor sequences for potential conversion events have not been found. Instead, it has been suggested that E_α and A_α genes, which most likely arose by gene duplication, have intrinsically different mutation rates due to different chromatin structures or sequence-specific mutagenesis, and that A_α may be freer to diverge to allow evolutionary experimentation (86).

REGULATION OF Ia EXPRESSION

Positive and Negative Regulation of Ia Surface Expression on Macrophages

Cell-surface expression of Ia antigens on macrophages is positively regulated by addition to the cell culture of supernatants of activated T cells (107–110). Biochemical analysis of the components of these supernatants, as well as analysis of the effect of cloned γ-interferon on Ia expression by macrophage cell lines, has shown that Ia induction is mediated by γ-interferon, a soluble product of stimulated T cells (110). γ-Interferon increases both I-A and I-E as well as H-2 expression (110) and appears to act at the level of mRNA transcription, since its addition to cultures of mouse macrophage cell lines results in a 10-fold increase in I region–encoded RNA (111); furthermore, treatment of human lymphoblastoid and myeloma cell lines with γ-interferon increases HLA-DR α and β chain mRNA (112).

Prostaglandins have been shown to negatively regulate expression of Ia antigens on uninduced cultured mouse macrophages and on macrophages induced by lymphokines (113). Glucocorticosteroids (114) and bacterial endotoxin LPS (lipopolysaccharide) (115) have also been shown to affect negatively the surface Ia expression on mouse epidermal Langerhans cells and macrophages. The data suggest that LPS inhibits γ-interferon regu-

lation of macrophage Ia-antigen expression by stimulating macrophage prostaglandin E2 production (115).

Nonexpression of a Surface I-E Molecule in Some Mouse Haplotypes

Mice of the b, s, f, and q haplotypes fail to express a serologically detectable I-E molecular complex (116). The defect in b- and s-haplotype mice appears to affect synthesis of only the I-E_α chain; E_α polypeptide is not detectable in the cytoplasm of lymphoid cells of these strains while normal levels of cytoplasmic I-E_β can be visualized by 2-D gels (116). The isolated E_β chain does not appear to be expressed on the cell surface in these strains, but the expression defect can be complemented, resulting in normal surface expression of a hybrid I-E complex in F1 hybrids between b or s haplotype strains and E_α-expressing strains (116). However, f- and q-haplotype mice synthesize detectable amounts of neither E_α nor E_β (116).

The molecular basis for the defects in E_α expression has been defined by characterization of E_α mRNA and DNA from these four strains (117). Northern blot analysis of b- and s-haplotype mRNA revealed that these strains fail to transcribe any E_α-hybridizing RNA, apparently owing to a deletion of approximately 650 base pairs in the promoter regions of their E_α genes, including the TATA box, RNA initiation site, and most or all of the first E_α exon (117). Mice of the f- and q-haplotypes transcribe E_α-hybridizing RNA. The f-haplotype mice were shown to synthesize normal amounts of an aberrant 2.8-kb species, and only small amounts of the normal 1.25-kb E_α RNA, while q-haplotype mice synthesize very low levels of only the aberrant-sized E_α-hybridizing RNA (117); this may reflect a defect in RNA splicing or stability (117).

The molecular basis for the generation of the defect in E_β expression in f- and q-haplotype strains has not been clarified, although Northern blot analysis of f- and q-haplotype RNA shows approximately 5% of the normal level of 1.3-kb E_β-hybridizing material in both cases (L. Mengle-Gaw, unpublished results). Whether this reflects low-level cross-hybridization of an E_β probe to A_β mRNA or low-level E_β transcription has not been determined. The only example of an $E_\alpha^+ E_\beta^-$ inbred strain is A.TFR5, an (A.CA × A.TL)F1 heterozygote intra–I region recombinant (118, 119). Mice of this strain fail to synthesize E_β polypeptide but synthesize normal amounts of cytoplasmic E_α (A. Begovich, personal communication) and express surface E_α at 10–20% of normal levels, as detected by lymphocyte absorption studies with anti I-E antisera (119).

In addition to the generation of I-E^- strains by Ia transcriptional defects and genetic mutation, it appears that preferential I-E subunit association

can lead to functionally I-E$^-$ strains. It has been shown by serological and biochemical analysis that E_α^u preferentially associates with E_β^u in F1 heterozygotes between the u haplotype and the b, k, or s haplotype (120). That this preferential association of I-E chains has a functional consequence has been shown for the response to pigeon cytochrome *c*; preferential chain association in (u × k)F1 hybrids results in the preferential expression of nonresponder $E_\alpha^u E_\beta^u$ complexes over responder $E_\alpha^u E_\beta^k$ complexes (94). This quantitative deficiency in cell-surface Ia expression correlates with a corresponding relative defect in antigen-presenting function (94).

It has been estimated that roughly 20% of wild mouse populations do not express cell-surface I-E molecules (121). There appears to be an association between this lack of surface I-E expression and the presence of t-chromosomes (122)—abnormal regions of chromosome 17 that affect normal genetics and development in the 40% of wild mice affected by the mutation (123). Recent genetic analysis of E_α expression in t-haplotypes revealed that over 50% of the t-haplotypes examined did not express an I-E complex (123). The analysis showed two types of defect to be responsible for nonexpression: Some strains transcribe E_α RNA but do not make functional protein; the majority of strains examined appear to have the same E_α promoter-region deletion as the b- and s-haplotype inbred strains (122). This data suggested that the E_α deletion is an old mutation, disseminated among certain H-2 haplotypes by the male segregation distortion associated with the t-chromosomes (122).

Regulatory Elements in Class-II Genes

Like many other eukaryotic genes, the 5′ flanking regions of class II genes contain TATA-box (124) and CCAAT-box (125) regulatory elements. In addition, the E_α and E_β genes also contain two short conserved sequences—an 8-mer and a 13-mer, separated by 19 or 20 base pairs—that are located 20 base pairs upstream of the TATA box in E_α (41) and 56 base pairs upstream in E_β (69). The conserved base composition of these sequences and their conserved separation have led to the suggestion that they may be involved in the regulation of the coordinate expression of class-II α and β genes (69). By analogy, high-level expression of the thymidine kinase gene depends upon two DNA elements upstream of the CCAAT box (126). Orientation-independent Ia-expressor cell specific–enhancer sequences, apparently distinct from the 8-mer and 13-mer, have recently been mapped on transfected E_β^d genomic fragments, by enhancer-dependent plasmid transformation of Ia-expressing and non-expressing cell lines, to a 2-kilobase region upstream of the E_β^d promoter (127).

EXPRESSION OF EXOGENOUS CLASS-II GENES IN CLONED CELL LINES

The past several years have seen rapid advances in the development of techniques for DNA-mediated gene transfer of immunoglobulin (128, 129), of class-I (130, 131) genes, and most recently of human and mouse class II genes (132, 133). Gene transfer and functional cell-surface expression of exogenous class-II genes in vitro, as well as examination of the effects of purposeful mutation (i.e. by exon shuffling or site-directed mutagenesis) of these genes on class-II gene function, provide great promise for clarifying the emerging structure-function relationships of the molecules involved in the generation of immune responsiveness.

The success of mouse class I–gene transfer and cell-surface expression in a mouse fibroblastoid cell line (Ltk^- cells) (130, 131, 134, 135) led to its use as the target cell in initial class II gene transfer experiments. Mouse L cells, which express no endogenous Ia molecules, have been successfully transformed with genomic clones for the I-A_α and I-A_β genes, and for I-A surface expression detected by anti-I-A monoclonal antibodies (133, 136). Biochemically and serologically, the exogenous I-A molecules expressed on these cells are indistinguishable from those expressed on the cells from which the clones were derived (133, 136). In view of the fact that mouse fibroblasts do not synthesize an invariant chain, I_i (133), it is interesting that exogenous I-A subunits exist at normal levels in the cytoplasm and are transported and expressed on the cell surface in the absence of any association with I_i, albeit at a lower level per cell than their endogenous counterparts, and also that introduction and expression of exogenous I_i do not increase surface I-A expression (133). These results indicate that I_i is not needed for I-A subunit assembly, and that it alone is not sufficient for Ia transport to the cell surface (133).

The development of cloned antigen-presenting B-cell (137) and macrophage (138) lines has made feasible the use of Ia-expressing lines as gene transfer targets for class II genes. Although there is now a report that I-A^k-transfected L cells can present keyhole limpet hemocyanin (KLH) to KLH-specific, I-A^k-restricted, helper T cells (136), it is generally thought that study of the function of transfected genes is most accurate using cells that normally express them, owing to the possible contributions of as-yet-unidentified cellular components in the physiological processes under investigation. To this end, a cloned B-cell tumor line that constitutively expresses Ia^d was transfected with the A^k_β gene and shown to serve as target for both allostimulation and GAT-specific activation of an I-A^k T-cell clone (139). The exogenous A^k_β gene is presumably expressed in association with endogenous A^d_α, and its recognition by an I-A^k–restricted T-cell clone

indicates either that GAT is presented by the A_β chain alone, or that A_α^k and A_α^d are functionally equivalent in this presentation system. Recently, a hamster B-cell line was transfected with mouse A_α and A_β genes (136). The serologically normal I-A molecules expressed on the cell surface were associated with hamster I_i and were capable of presenting KLH to T-helper hybridomas. Transfection of a cloned E_β^b gene into H-2^d B-cell lymphoma and macrophage tumor lines and then examination of transformed cells with appropriate monoclonal antibodies showed the transfected gene to be expressed at the cell surface of both cell types, in association with endogenous E_α^d; so the gene was able to present antigen to restricted T-cell hybridomas (140). While the transfected gene was expressed constitutively in the B-lymphoma line, only transfected macrophages treated with γ-interferon expressed E_β^b, suggesting that the regulatory element(s) necessary for γ-interferon induction was associated with the transfected gene (140).

Exon shuffling—the creation of hybrid genes in vitro by joining certain exons from one gene with complementary exons from another gene—has been performed between class I genes; the resulting mutant hybrids were expressed exogenously in transfected cells (131, 141). With the foundation laid for class II genes (now that they can be stably introduced into physiologically appropriate differentiated cells in vitro and expressed on the surface in a functional way), the really interesting experiments can and have begun. Imaginative constructs of hybrid genes composed of complementary genetic segments from class I and class II genes (142) or from different haplotypes will certainly be transfected and their functions in antigen presentation analyzed to address the questions of haplotypic specificity and to identify the molecular components of Ia molecules essential for MHC restriction.

I-J: THE ELUSIVE GENE

The I-J subregion was originally defined serologically and mapped between I-A and I-E by reciprocal alloantisera raised between strains B10.A(3R) and B10.A(5R), which are inbred congenic recombinants with crossovers between I-A and I-E (143, 144). Alloantisera and monoclonal antibodies raised in I-J-disparate strains recognize polymorphic I-J-encoded determinants expressed on suppressor T cells, possibly as part of the suppressor–T cell antigen receptor; they also recognize the soluble suppressor factors secreted by these cells (143–150). Biochemical characterization of the I-J determinants has proceeded slowly, owing in part to the apparently very low level of expression of these determinants on the relatively small fraction of suppressor T cells. Anti-I-J monoclonal antibodies appear to recognize a 25,000-dalton polypeptide (148).

Using restriction fragment polymorphisms to map crossover points in inbred congenic strains that have recombination events between the I-A and I-E subregions, initial molecular analysis of the I region mapped the I-J locus to 3.4 kb between I-A and I-E, a region that included the 3′ half of the E_β gene (39). Molecular cloning of this 3.4-kb region from ten parental and intra–I region recombinant strains and fine mapping of restriction site polymorphisms have narrowed the estimate of the distance between crossover points separating boundaries of I-A and I-E to 2.0 kb, contained entirely within the $\beta1$-$\beta2$ intron and $\beta2$ exon of the E_β gene (151).

These results have led to much speculation concerning the true identity and chromosomal location of the I-J locus. It is apparent from the restriction site mapping data that the I-J molecule is not a posttranscriptionally or posttranslationally modified form of the complete E_β gene product, an idea suggested by the first molecular data mapping I-J to a region overlapping the E_β locus (39). In fact, 2.0 kb is sufficient to encode a 25,000-dalton polypeptide (assuming only several very short introns), and the I-J molecule may be read from the DNA strand opposite that which encodes E_β (151). Alternatively, it has been suggested that this 2.0-kb region encodes only part of the I-J determinant, perhaps one or several polymorphic exons joined by DNA rearrangement or alternative RNA splicing to additional exons encoded elsewhere (151). All of these explanations are refuted by experiments showing that cloned DNA from this region fails to hybridize to RNA from $I\text{-}J^+$ T-suppressor cell lines (152).

Alternative explanations have been put forth to account for the possibility that I-J may only appear to map between I-A and I-E by serological and classical genetic analysis, while in fact it is encoded elsewhere in the I region or even outside the MHC altogether. The segregation of I-J with the I region may in fact be explained by the presence, in the recombinant strains used to map I-J, of triple crossovers between I-E and an I-J locus mapping many centimorgans away (151). In the absence of multiple genetic markers spanning the MHC, this possibility cannot be ruled out. If an I-J polypeptide is encoded outside the MHC, perhaps on another chromosome, its expression may be regulated by a gene encoded within the 2.0-kb H-2 region.

Recently, evidence was put forth indicating that 3R and 5R differ not only at H-2 loci, but at non-H-2 loci as well, and that T cell–surface expression of $I\text{-}J^k$ depends not only on I region sequences but also requires the complementation of at least two genes, one of which maps to chromosome 4 of the mouse, the Jt gene, while the other most likely maps to the I-E subregion of the H-2 complex (153). The finding that binding of anti-$I\text{-}J^k$ monoclonal antibodies to cell-surface $I\text{-}J^k$ determinants is obliterated by treatment with α mannosidase indicates that the I-J determinant involves a glycoprotein; it has been suggested that the function of the Jt gene product

may be to glycosylate an I-region product (154). I-J^k determinants may be present on a Jt glycoprotein that may form part of the inducible T-cell receptor for self-I-E (153). A related explanation has suggested that I-J may be a sort of serological artefact: Antisera raised in I-J-disparate strains may contain antibodies that recognize self-E_β T-cell receptors (155), antiself idiotypes (156). These possibilities would predict that the I-J determinant would appear to map by serological analysis to the E_β locus. The polymorphic nature of I-J determinants would be explained by presuming that the crossovers in 3R and 5R occurred so as to affect differentially the structures of their E_β products; that would in turn elicit different self-receptor structures. Finally, the Jt gene product may be enzymatically active specifically in T cells, modifying the H-2-encoded product (153). This seems unlikely (*a*) because H-2^q mice are I-E_β^- but I-J^+ and (*b*) in light of the failure to find E_β-hybridizing RNA in I-J^+ cells. The data now emerging on I-J serve as much to confuse and confound as to illuminate; past and present efforts to solve of riddle of I-J foretell that the eventual answers will be complex ones.

CONCLUSIONS

The past decade has seen significant advances in our understanding of the molecular nature and functions of immune response genes. Recombinant DNA technology has afforded an impressive dissection of the genetic structure and organization of mouse and human class-II antigens. However complete our understanding of MHC genetics, real understanding of the mechanistic basis underlying immune responsiveness waits for the coordination of molecular and cellular findings. Although many questions have been answered, many more have been raised, and hopefully the next decade will provide satisfying answers to these: in particular, a description of the three-dimensional structures of Ia, foreign antigen, and antigen-specific T cell–receptor molecules. The real payoff will come when we can extrapolate what we've learned about Ir-gene function to be able to manipulate that function, as well as to apply it in other biological systems.

"Brains first and then Hard Work.
That's the way to build a house," said Eeyore.
A. A. Milne

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CONTENTS

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THE EFFECTS OF CYCLOSPORIN A ON THE IMMUNE SYSTEM[1]

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INTRODUCTION

The cyclosporins were originally discovered in 1970 by workers at Sandoz Ltd. in Switzerland who were attempting to identify new antifungal agents (1). The crude extracts of two new strains of fungi imperfecti, *Cylindrocapon lucidum* Booth and *Tolypocladium inflatum* Gams, demonstrated a narrow spectrum of activity in vitro and only marginal fungistatic activity in vivo mainly against clinically irrelevant organisms. However, the antifungal activity was accompanied by an unusually low degree of toxicity, which prompted the investigators at Sandoz to submit the compound to a limited pharmacological screening program. In 1972, Borel discovered that the fungal extract was capable of markedly inhibiting hemagglutinin formation against sheep erythrocytes in vivo but appeared to be selective in its immunosuppressive effects because it had no effect on the survival of mice that had been inoculated with the L1210 leukemia line. This observation formed the basis for a series of extensive studies of the effects of cyclosporin (CY A) on the immune system.

In 1973, cyclosporin A was purified from the fungal extract, and in 1975 complete structural analysis was performed (2). CY A was successfully synthesized in 1980. CY A (M_r 1203) is a neutral, hydrophobic, cyclic peptide composed of 11 amino acid residues all having the S-configuration of the natural L-amino acids with the sole exception of the D-alanine in position eight, which has the R-configuration. Ten of the amino acids are known aliphatic amino acids while one of the amino acids had never

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previously been isolated or known in its free form; this one is referred to as the C9-amino acid because it has nine carbon atoms. The other fungal metabolites related to CY A are named as follows: [ala^2]-cyclosporin (CY B), [thr^2]-cyclosporin (CY C), and [Val^2]-cyclosporin (CY D).

The early studies of Borel and coworkers (3, 4) clearly established the basis for the enthusiasm for CY A as a selective immunosuppressive agent that appeared to act on only a defined subpopulation of immunocompetent cells. CY A was found to suppress hemagglutinin formation as well as direct and indirect plaque-forming cell (PFC) responses against sheep erythrocytes. Skin allograft survival was prolonged by CY A and a delayed onset of death was seen in an experimental model of lethal graft-vs-host disease (GVHD). CY A reduced the incidence of polyarthritis in rats following administration of Freund's adjuvant and also suppressed disease activity in the rat model of experimental allergic encephalomyelitis (EAE). The T-cell specificity for the immunosuppressive effects of CY A was suggested by the failure of CY A to suppress antibody response to lipopolysaccharide (LPS) antigens in vivo in nude mice. In contrast to other immunosuppressive agents like azathioprine, CY A appeared to be free of myelotoxicity and did not impair the number or proliferative capacity of hematopoietic stem cells.

In this review I summarize the many experiments performed over the past ten years describing a host of immunologic events that seem to be altered selectively by CY A. I emphasize to the experimental immunologist not only that CY A is a drug useful in prolonging allograft rejection and treating certain autoimmune diseases but also that it is an important immunologic reagent that may play a critical role in dissecting various complex immunologic phenomena.

EFFECTS OF CY A ON T-CELL ACTIVATION

Interleukin-2 Production and Responsiveness

Although the early experiments of Borel (3) demonstrated that CY A readily inhibited the proliferative response to mitogens such as concanavalin A (Con A) or phytohemagglutinin (PHA), it was not until the concept of the interleukin cascade was proposed that further advances could be made in defining the site of action for the inhibitory effects of CY A on T-cell proliferation. The T-cell activation process in response to stimulation by mitogens can be divided into two steps. The first step involves the induction of cell-surface receptors for the growth factor, interleukin-2 (IL-2), while the second step involves the production of the growth factor.

Larsson (5) was the first to study whether CY A blocked the induction of IL-2 receptors or the production of IL-2. Murine spleen cells were incubated for 4 hr at 37°C in the presence of Con A and in the presence or

absence of CY A. The cultures were then washed four times in medium containing α-methyl-mannoside to remove cell-bound Con A and then tested for responsiveness to IL-2. Control spleen cells cultured with Con A but not in CY A responded to IL-2 while spleen cells treated with Con A in the presence of CY A failed to respond to IL-2. The production of IL-2 in response to Con A over a 24-hr period was unaffected by CY A.

Bunjes et al (6) reached exactly the opposite conclusion in their studies of the effects of CY A on the cellular events involved in the induction of cytotoxic T lymphocytes (CTL) in the murine mixed-leukocyte reaction (MLR). CY A markedly inhibited the induction of CTL in the MLR and also totally inhibited IL-2 production by spleen cells stimulated by Con A. When exogenous IL-2 was added to CY A–treated MLR cultures, highly reactive CTL could be induced. The conclusions drawn from these studies were that (*a*) CY A exerted its major inhibitory effect by blocking IL-2 production, and (*b*) it had little, if any, inhibitory effect on the induction of IL-2 receptors on the Ly-2$^+$ precursor of the major histocompatibility complex (MHC), class I–restricted, alloreactive CTL.

The effects of CY A on IL-2 production and responsiveness were further complicated by the studies of Palacios (7), which demonstrated that both steps in the T-cell activation process may be susceptible to inhibition by CY A. CY A–treated, Con A–stimulated human lymphocytes failed to produce IL-2, or to respond to IL-2, and also were unable to absorb the growth factor. He also demonstrated that CY A inhibited the production of the monokine, IL-1, from macrophages by acting on OKT4$^+$ T lymphocytes; pretreatment of the T cells with CY A abrogated IL-1 production, whereas pretreatment of the macrophages had no effect; the response to LPS stimulation was not changed by the addition of CY A to the cultures.

The effects of CY A on human T-lymphocyte responses were not limited to stimulation by mitogens; Palacios (8, 9) was readily able to inhibit the proliferative response induced by self-Ia in the autologous MLR (AMLR) by the addition of CY A to the cultures. The mechanisms involved in AMLR inhibition were identical to those seen in response to Con A. T cells in CY A–treated AMLR cultures failed to produce, respond to, or absorb IL-2. CY A also inhibited the production of helper-factor for B cells as well as the generation of suppressor and CTL functions in the AMLR. Taken together these findings suggested to Palacios that CY A might exert its inhibitory function by blocking the T-cell receptor that recognizes self-Ia.

Dos Reis & Shevach (10) employed a strategy similar to that used by Bunjes et al (6) to study the effects of CY A on several different models of T-cell activation in the guinea pig. All of the assays were performed both in the presence and absence of high concentrations of IL-2-containing supernatant fluids, allowing one to differentiate the effects of CY A on growth-factor production from the effects it produced on induction of the IL-2

receptor. CY A inhibited different modes of guinea pig T-lymphocyte activation through different mechanisms depending on the type of stimulus and the differentiation state of the responding T cell. For example, Con A–induced proliferation was inhibited primarily by a blockade of IL-2 production, as a substantial reconstitution of the proliferative response could be achieved by addition of IL-2 containing supernatants to the culture. In contrast, CY A suppressed the AMLR by blocking both IL-2 production and the induction of IL-2 responsiveness. The allogeneic MLR was inhibited solely by a blockade of IL-2 production as addition of exogenous IL-2 completely restored the T cell–proliferative response.

Several difficulties in the interpretation of these findings should be emphasized. First, the effects of IL-2 on the induction of IL-2 responsiveness were analyzed functionally; the presence or absence of IL-2 receptors was not specifically measured in ligand-binding studies, absorption experiments, or with monoclonal antibodies to the IL-2 receptor. Second, an unpurified supernatant obtained from mitogen-activated, guinea-pig lymph node cells was used as a source of IL-2, and one cannot exclude the possibility that some of the effects mediated by this supernatant could have been produced by factors other than IL-2. In any case, the IL-2 containing supernatant restored some, but not all, of the T lymphocyte–proliferative responses inhibited by CY A.

IL-2 Receptor Expression

The anti-Tac antibody identifies the receptor site for IL-2 on activated human T cells and inhibits IL-2-dependent proliferation as well as the binding of ^{3}H-IL-2 (11). Miyawaki et al (12) used the immunoperoxidase method to assess the effects of CY A on the expression of the Tac antigen by Con A– and PHA-activated human lymphocytes. Although both proliferation and blast transformation were inhibited, the expression of Tac was not inhibited. Furthermore, T cells recovered from cultures of mitogen-stimulated, CY A–treated cells could also absorb IL-2 activity in a manner quantitatively similar to that of T cells from untreated cultures. When T cells were incubated with Con A and CY A for 24 hr, the presence of CY A did not inhibit the induction of responsiveness to IL-2; such T cells were fully responsive to IL-2 even if the secondary cultures were performed in the continued presence of CY A.

A similar series of studies was carried out in the mouse by Lillehoj et al (13), who used a rat monoclonal antibody, 7D4, that is directed to an epitope on the murine IL-2 receptor distal to the IL-2 binding site (14). CY A produced an 80–85% inhibition of IL-2 production and also completely inhibited the proliferative response to Con A. The addition of an excess of IL-2-containing supernatant fluid at culture initiation had only a slight

effect on enhancing responses significantly above control cultures. This failure of exogenous IL-2 to reconstitute the proliferative response suggested that CY A exerted its inhibitory effects in a more complex manner than merely by inhibiting lymphokine production. When T cells were cultured for 72 hr with Con A in the presence of CY A, a dose-dependent inhibition of IL-2 receptor expression was seen when the cells were analyzed on the FACS (flourescence activated cell sorter) with antibody 7D4. The addition of exogenous IL-1 and/or IL-2 did not abrogate this CY A–mediated inhibition of IL-2-receptor expression. Surprisingly, CY A had only minimal effects on IL-2-receptor expression 24 hr after Con A stimulation. Furthermore, the effects of CY A on the induction of IL-2 responsiveness were puzzling. T cells isolated from cultures after either one or three days of stimulation failed to respond to exogenous IL-2 in spite of the fact that the cells after one day of stimulation even in the presence of CY A bore near normal levels of IL-2 and transferrin receptors.

It is very difficult to reconcile all the observations that have been made of the effects of CY A on the production of IL-2, the expression of IL-2 receptors, and the induction of IL-2 responsiveness. In contrast to the initial experiments of Larsson (5), almost all subsequent studies have demonstrated inhibition of IL-2 production by CY A, and molecular approaches have begun to determine the specific site of this inhibitory effect (see below). The heterogeneity of experimental findings on IL-2 responsiveness suggest that the effects of CY A on IL-2 receptor expression and function are complex. In particular, the experiments in man (12) and in the mouse (13) appear to yield exactly opposite conclusions. Although these studies were performed on two different species, they both used well-characterized monoclonal antibodies to the IL-2 receptor. One difficulty with the study of Miyawaki et al (12) is that the presence or absence of the Tac antigen was assayed by the immunoperoxidase method, which does not lend itself to a quantitative analysis of membrane antigen expression. The FACS profiles obtained with a log amplifier clearly allow a more precise quantitative comparison of receptor expression on different cell populations. It is possible that human T lymphocytes maintain over three days of culture a low, but not normal, level of Tac expression even when cultured in the presence of CY A.

The differences between the human and mouse studies of the effects of CY A on the induction of IL-2 responsiveness are very difficult to resolve. One possibility is that CY A impairs the production of other lymphokines that are required for the induction of IL-2 responsiveness and that the concentration of these lymphokines in the supernatants used in the mouse studies was insufficient to reconstitute the response, whereas it may have been present in large amounts in the supernatants used in the human

studies. Alternatively, CY A may exert differential effects on the lymphocytes of different species on the expression of other cell-surface antigens that are required to process the IL-2 growth signal or to convert the IL-2 receptors from a low-affinity to a high-affinity state. At present, it is probably premature to conclude that CY A blocks a single step in T-lymphocyte activation. It is much more likely that CY A interferes with several distinct steps in the T cell–activation process, and the specific step inhibited varies with the species and specific stimulus used to induce the activation process.

Effects on the Production of Other Lymphokines

The effects of CY A on the production of a number of lymphokines other than IL-2 have also been studied in vitro. Thomson et al (15) demonstrated that the generation by activated guinea-pig T lymphocytes of migration inhibition factor (MIF)—the factor that induces macrophage procoagulant activity (MPCA)—and of lymphocyte derived chemotactic factor (LDCF) were both impaired in the presence of CY A. However, CY A had no direct effect on the migration of peritoneal cells from capillary tubes or on the responses of macrophages to preformed MIF, to LDCF, or to the lymphokine that induces MPCA. Similar results were reported by Helin & Edgington (16), who demonstrated that CY A inhibited the generation of MPCA when added at the initiation of a two-way MLR between pairs of human donors.

The effects of CY A on interferon (IFN) production have also been analyzed by several groups (17–19). IFN α/β production by virus-infected human or mouse lymphocytes and fibroblasts was not inhibited by the addition of CY A. Since virus-induced IFN α/β production has usually been associated with B lymphocytes or macrophages, this result is consistent with the selective effect of CY A on T lymphocytes. In contrast, CY A totally inhibited IFN γ production by human or mouse lymphocytes that were stimulated by mitogen or alloantigen. It is likely that the effects of CY A on inhibition of IFN γ production are not secondary to its inhibitory effects on IL-2 production because even when CY A was added to cultures 24–48 hr after culture initiation, IFN γ production at 3–5 days was still inhibited.

Effects on Early Events in T-Cell Activation

In general, CY A must be present at culture initiation or must be added shortly thereafter in order to observe an inhibition of mitogen-induced, T-cell activation. Kay & Benzie (20) have measured protein synthesis 20 hr after lectin activation and found that CY A inhibited the response induced by Con A to a greater extent than the response induced by PHA. Similarly, marked inhibition of ^{3}H-uridine uptake and incorporation into RNA at

24 hr after activation with Con A was seen, while the PHA response was affected much less. Kay et al (21) also examined the response of pig lymphocytes to the calcium ionophore, A23187. Activation of lymphocytes by this agent might be expected to bypass some of the steps used by lectins which presumably mediate their effects by binding to T-lymphocyte glycoproteins. A23187 was found to produce a substantial increase in the rate of uridine uptake in the first hour after its addition and CY A produced a marked inhibition of this rise in uridine uptake as well as a substantial inhibition of the rise in ^{35}S-methionine incorporation seen 23 hr after stimulation by A23187.

Although attention has been focused on determining which of the early events in lymphocyte activation are inhibitable by CY A, a well-defined activity that was not susceptible to inhibition by CY A would perhaps allow one to pinpoint its site of action. One of the earliest events in lectin-induced transformation is considered by many (22) to be a rise in cytoplasmic calcium $[Ca^{+2}]i$. Metcalfe (23) found that the Con A–induced rise in $[Ca^{+2}]i$, seen in the first few minutes after stimulation, was neither prevented nor enhanced by preincubation of the responding T cells in CY A. The conclusion drawn from this study was that CY A must act at a later step in the activation process.

Sugawara & Ishizaka (24) have reported that CY A markedly inhibited the proliferative response of human lymphocytes induced by Con A and to a lesser degree the response induced by PHA. However, CY A had no effect on the response of human T lymphocytes to stimulation by the phorbol ester, phorbol myristate acetate (PMA). Although PMA is not mitogenic for resting murine T lymphocytes, we have recently observed (E. M. Shevach, unpublished observations) that the combination of PMA and purified IL-2 induces a large proliferative response in resting murine splenic T lymphocytes, which is totally resistant to inhibition by concentrations of CY A that inhibit the Con A response even in cultures supplemented with an equivalent concentration of IL-2. Taken together, these observations suggest that there may be an alternative pathway for T-cell activation, perhaps mediated by the receptor for phorbol esters, which bypasses the CY A sensitive steps in T-cell activation. Similar findings will be discussed in the section on B-cell activation.

Effects on Cloned T Cells

One of the major difficulties in defining the site of action of CY A is that the majority of experiments have been performed with uncloned heterogeneous populations of cells stimulated with polyclonal mitogens. Orosz et al (25) analyzed the effects of CY A on a panel of alloantigen-stimulated T-cell clones with three distinct phenotypes: (*a*) helper T cells that proliferate and

secrete IL-2 when stimulated with alloantigen; (*b*) conventional cytolytic T-cell clones that fail to proliferate to alloantigen unless exogenous IL-2 is provided; and (*c*) helper T cell–independent T-cell lines that are cytolytic but also proliferate when cultured with specific alloantigen even in the absence of exogenous IL-2. CY A markedly inhibited the T cell–proliferative response induced by specific alloantigen while having no effect on the response of the clones to IL-2. Exogenous IL-2 did not reverse the CY A–mediated inhibition of antigen-induced proliferation. When helper T cell or helper T cell–independent cytolytic clones were cultured with exogenous IL-2 plus specific alloantigen, additive or synergistic proliferative responses were observed, but only the antigen-driven component of these responses was inhibited by CY A. CY A also inhibited the proliferative responses of conventional cytolytic T-cell clones but only in the presence of alloantigen and exogenous IL-2; again, the IL-2 driven component of the response was totally unaffected by CY A. These studies support the view that CY A is capable of inhibiting the generation of CTL during the course of an MLR both by blocking helper T-cell function as well as by blocking the antigen-driven component of cytolytic T cell–precursor proliferation. It is very unlikely that any of the inhibitory effects of CY A are mediated by the inhibition of the recognition of specific alloantigen by the T-cell receptor because CY A had no effect on the ability of cloned cytolytic T cells to lyse targets.

Further studies of this group (26) have demonstrated that helper T-cell clones failed to secrete IL-2 when stimulated by alloantigen in the presence of CY A and that CY A blocked the secretion of IL-2 and IL-3 from these clones following Con A stimulation. The effect of CY A on IL-2 receptor expression by these cloned lines was tested indirectly in IL-2-absorption assays. Cytolytic clones were found to have an increased number of IL-2 receptors, as was demonstrated by an enhanced capacity to absorb IL-2 after stimulation with the appropriate alloantigen. This antigen-driven increase in absorption capacity was not blocked by a concentration of CY A that was capable of inhibiting the antigen-induced proliferative response.

Effects on T-Cell Hybridomas

A number of bifunctional CTL hybridomas that grow constitutively in culture without exogenous growth factors have been identified by Kaufmann & Rosenberg (27). When challenged with specific alloantigen, they develop CTL activity and secrete IL-2. These lines have some of the characteristics of memory CTL in that they require a 2–3-hr incubation period with specific antigen before specific lytic activity can be detected. Alloantigen-induced IL-2 secretion was completely inhibited when CY A was added at the same time as alloantigen. CY A also inhibited the specific

lytic activity of nonactivated hybridomas very efficiently but to a much lesser degree inhibited lysis by cells that had been preactivated by specific antigen. Nonspecific killing induced by stimulation with Con A was also inhibited by CY A. Thus, CY A appears to block one or more steps in the process of antigen-receptor interactions needed for induction of differentiation and to have no effect on the mechanisms involved in cell lysis.

The effects of CY A on IL-2 secretion by a series of T-cell hybridomas specific for protein antigens in association with MHC–class II product have also been examined (P. Wassmer, E. M. Shevach, unpublished observations). The secretion of IL-2 by these lines in response to antigen as well as to Con A was inhibited by CY A. Because Con A stimulation of these hybridomas is performed in the absence of any added accessory cells, it is very likely that CY A is suppressing T-cell activation by acting directly on the T-cell hybridoma rather than by mediating its effects by acting on a non-T accessory cell.

Lastly, the availability of both T-cell lines with defined biologic function as well as cDNA probes specific for a number of lymphokine genes now offers an opportunity to examine the effects of CY A at a molecular level. Kronke et al (28) have recently used a cloned cDNA probe for human IL-2 to investigate the effects of CY A on IL-2 mRNA levels in a cloned line of the Jurkat leukemia. No IL-2 mRNA could be detected in uninduced Jurkat; but following induction with PHA and PMA, IL-2 mRNA was detected in cytoplasmic dot-blot hybridizations after 1 hr and the peak was observed at 6 hr. Activation of Jurkat T cells in the presence of CY A prevented detection of IL-2 mRNA.

EFFECTS OF CY A ON ALLOREACTIVITY

In Vivo Studies

The role of CY A as a potent suppressor of organ allograft rejection has been demonstrated for many different organ allografts in a wide variety of species (29, 30). Probably the most intriguing observation made with CY A is that for many but not all of the organs transplanted a limited course of CY A leads to very prolonged allograft survival. The critical questions that remain to be answered are: What is the mechanism by which a brief treatment with CY A leads to long-term graft acceptance, and how can this situation be achieved in man?

Concerning the mechanism of allograft acceptance following cessation of CY A therapy, the most insightful series of experiments have been performed in the rat model, where rejection of either kidney or heart is readily suppressed by a short course of CY A. Nagao et al (31) grafted PVG-strain rats with hearts from DA-strain donors and administered 14 days of

CY A immunosuppression. A secondary challenge of skin from DA or WAG-strain rats was then used to investigate the degree and specificity of the unresponsive state induced by CY A. Three distinct stages could be identified. Stage 1 coincided with the period of CY A treatment and represented a period of profound unresponsiveness of a nonspecific nature. Compared to a DA-skin graft, a WAG-skin graft placed on a PVG recipient of a DA heart was equally protected from rejection; rejection of skin was rarely followed by loss of a heart graft. It should be emphasized that the lack of reactivity did require the presence of a heart graft, because skin grafted in the absence of a heart transplant enjoyed only a mild prolongation of survival following cessation of CY A therapy. In stage 2, nonresponsiveness declined and skin-graft survival was not prolonged, with rejection of a DA-skin graft inevitably followed by loss of the DA-heart graft. Some DA hearts were even lost after rejection of WAG skin. In stage 3, which developed between the eighth and sixteenth week after transplant, specific non-responsiveness to skin grafts from the donor heart strain was seen. Such grafts survived for a prolonged period although they were eventually rejected chronically; in contrast, the third party WAG-skin graft was rejected in normal acute first-set fashion. The third stage was very stable, as these investigators failed to induce the rejection of the heart graft either by skin grafts or by transfer into recipients of normal or sensitized lymphocytes. The major question raised by this series of studies is whether stage 2 has to be induced in order to develop the third stage of donor-specific stable tolerance. If CY A had been administered for 16 weeks rather than 2 weeks, would stage 1 nonresponsiveness have become stage 3?

This group has also investigated the antigen dependence of CY A–induced allograft acceptance (32). WAG recipients were grafted with DA hearts and treated with CY A for 14 days. The heart graft was removed 14–33 days after its implantation; the animals were rested for four weeks and then tested for the presence of the tolerant state by transplanting a second DA heart into the recipients. Almost all second grafts were rejected; thus it was demonstrated that the mechanism by which the graft is retained after cessation of CY A therapy is a dynamic one requiring the presence of donor antigen for maintenance.

In Vitro Studies: Human Lymphocytes

The successful use of CY A in vivo in both experimental animals and man to prolong allograft rejection has prompted a large number of in vitro studies to investigate the mechanisms involved in the tolerant state. Most studies have concentrated their efforts on the identification of specific suppressor T lymphocytes either induced by CY A or spared from death in the presence of CY A. Although the in vitro models may not represent a correlate of the

tolerant state induced in vivo, these analyses may throw some light on the mechanism by which CY A inhibits T-lymphocyte activation in general.

Hess & Tutschka (33) were the first to demonstrate that alloantigen-induced suppressor T cells were relatively resistant to the effects of CY A in culture. Thus, at a concentration of 1 μg/ml of CY A, CTL activity was absent, whereas both specific and nonspecific suppressor-cell activities were similar to those seen in control cultures. It should be emphasized that specific and nonspecific suppressor-cell activities were never greater in CY A–treated cultures than in control-MLR cultures, which suggests that CY A did not induce suppressor cells but rather allowed or permitted their expression only in the presence of the alloantigen. Although suppressor-cell activity was found in cultures without any demonstrable proliferative response, concentrations of CY A that did allow some proliferation also allowed an amplification of suppressor-cell activity. Alloantigen-suppressor cells present in control and in CY A–treated, primary MLR cultures were both radiation sensitive (1500r). Autologously primed T cells cultured with CY A did not result in suppressor cell activity; thus, it is unlikely that the suppression observed in the second test cultures was due to carryover of CY A.

Further characterization (34, 35) of the suppressor-cell population induced by CY A demonstrated that lymphocytes isolated from CY A–treated, primary MLR cultures gave only minimal proliferative responses when rechallenged, and no CTL activity could be measured. Such cell populations had a two- to five-fold increase of suppressor-cell activity. Rechallenge with third party, unrelated alloantigens resulted in the induction of CTL. Nylon wool fractionation of the primed lymphocytes from CY A–treated cultures restored the capacity to generate CTL effector cells in response to the original alloantigen. These data are most compatible with the presence of a population of nylon wool–adherent suppressor cells that develop in both control- and CY A–treated MLR cultures and that function to regulate the MLR and to inhibit the induction of CTL.

Studies by Mohagheghpour et al (36) involved the performance of 12-day MLR cultures in the presence of CY A with subsequent isolation of the various T-cell subsets with monoclonal antibodies; each subset was then tested for its ability to inhibit a second fresh MLR in the absence of CY A. The T suppressors utilized in these studies were irradiated after their activation in primary cultures and only stimulator-specific suppression was observed. Furthermore, responses to cells that shared at least one DR allele with the original stimulator were inhibited regardless of their HLA-A,B types. Both $T8^+$ and $T4^+$ subsets derived from CY A–treated cultures exhibited suppressor activity. However, the $T4^+$ suppressor cells only induced potent suppression if the indicator MLR contained fresh $T8^+$ cells,

and they had little effect on fresh MLR's with purified T4 responders. In contrast, $T8^+$ cells derived from CY A–treated cultures suppressed the response of $T4^+$ cells in the absence of fresh $T8^+$ cells. It also appeared that in the T4 population the CTL-inducer functions were CY A sensitive, but the suppressor-inducer functions were CY A resistant.

In Vitro Studies: Rodent Lymphocytes

Although the effects of CY A on the human MLR have been studied extensively, surprisingly few studies have been performed in animal models. Dos Reis & Shevach (10) demonstrated that CY A–treated guinea pig–MLR cultures contained a suppressor-cell population that was able to inhibit the primary MLR response. The suppressor cell in this model was radio-sensitive, which ruled out the possibility that the suppression resulted from carryover of CY A into the test MLR cultures. T cells primed in the absence of CY A did not show any suppressive activity; in addition, T cells primed in the presence or absence of CY A to syngeneic stimulators had no suppressive activity. The presence of alloantigen was required in addition to CY A to generate suppressors.

Dos Reis & Shevach (37) also demonstrated that the CY A–induced T suppressor cells, after overnight culture with stimulator cells but in the absence of CY A, secreted a factor with marked suppressive activity in the MLR. The factor would only suppress a primary MLR of responder-T cells that were derived from the same strain as the factor producer, but it demonstrated no specificity for the stimulator cell. Possibly, such a factor is associated with shed stimulator Ia antigens in such a manner that only the responder-specific binding site is available for interaction with the T cell.

Wang et al (38, 39) have performed an extensive series of studies on the effect of CY A on alloreactivity in the mouse. They confirmed that the addition of CY A to the mouse MLR inhibited both proliferation and the generation of alloreactive CTL. Administration of CY A resulted in a failure of the induction of CTL in vivo, and lymphocytes from these treated animals were incapable of being activated in an in vitro MLR upon reexposure to the same alloantigens. When lymphocytes obtained from mice that had been alloantigen sensitized and CY A treated were then mixed with fresh normal lymphocytes in vitro, a marked dose-dependent suppression of the generation of CTL activity was observed. These suppressor cells were not detected in mice receiving CY A alone, which indicates that CY A did not induce but rather permitted the expression of suppressor cells that were generated during the course of allosensitization. The suppressor cells in this system were completely antigen-nonspecific, but the possibility remains that they represented a population of

suppressor-inducer cells that acted to generate suppressor cells specific for the sensitizing antigen.

In summary, a large number of studies have demonstrated that suppressor cells with different properties can be generated in vitro during the course of sensitization to alloantigen in the presence of CY A. It is difficult to translate the results of these in vitro studies to the in vivo situation, but one must assume that certain of these suppressor populations are responsible for the long-term immunosuppression induced by CY A in vivo. Certain questions remain unresolved. Why can CY A be given only as a short course of therapy with certain allografts in some species but not others (compare renal allografts in rat and man)? Why are some grafts (skin) always rejected when CY A therapy is stopped, irrespective of the species? Are the suppressor cells too weak? Are different types of T suppressor cells involved in the maintenance of allograft tolerance with different types of grafts?

Two important areas remain for future study. One should be the use of CY A by the experimental immunologist to study T suppressor cells to both alloantigens and to soluble protein antigens. This area has not yet been exploited. Second, CY A should be used to generate suppressor T-cell clones or hybridomas; this should facilitate the isolation of large quantities of cell-free supernatants that by themselves might be capable of inducing and maintaining specific transplantation tolerance in vivo.

EFFECTS OF CY A ON B-LYMPHOCYTE ACTIVATION

Studies on Murine B Lymphocytes

The early studies of Borel (3) suggested that CY A did not appear to affect B-cell function because antibody production to LPS was not reduced in nude mice. The in vivo effects of CY A on B-cell function were analyzed in much greater depth by Kunkl & Klaus (40). CY A (50 mg/kg) given subcutaneously on days 1–3 totally abolished the day-4 PFC response to DNP-Ficoll. In striking contrast, five days of treatment with CY A produced a two- to five-fold enhancement of the response to DNP-LPS. CY A was most effective when given around the time of immunization, whereas on days 2–4 it was no longer effective.

CY A given on days 1–6 totally ablated the primary response to DNP-KLH (day 7 assay), while having no effect on the secondary response (day 4 assay). These data suggested that primed, thymic-dependent (TD) B cells and primed helper T cells were resistant to CY A. However, it was impossible to tell whether the inhibition of the primary response reflected

helper T cell or B-cell inactivation by CY A. Mice were therefore primed with KLH and four weeks later boosted with DNP-KLH. Three days of treatment with CY A produced marked suppression of the peak IgM (day 3) and IgG (day 5) responses in mice not preimmunized with KLH. In contrast, the drug had no effect on the day-3 IgM or the day-5 IgG responses in KLH primed mice. These results are most consistent with the possibility that all three—primary TD B cells, secondary TD B cells, and B cells responsive to TI-1 antigens—are CY A resistant. Virgin T helper cells and those B lymphocytes responding to TI-2 antigens, the subset lacking in CBA/N mice, are exquisitely sensitive.

Dongworth & Klaus (41) examined the effect of CY A on the in vitro responses of B cells to a number of polyclonal activators. The proliferative response of B cells to anti-Ig antibodies was highly susceptible to inhibition by CY A, whereas the proliferative response to LPS was resistant. In general, the B-cell response to anti-μ stimulation was inhibitable by lower concentrations of CY A than that needed to suppress the T-cell response to Con A. This observation raised the possibility that the effects of CY A on B-cell proliferation were indirect and were mediated by the activity of CY A on T cells that contaminated the B cells and that were required for B-cell growth. However, depletion of either T cells or macrophages had no effect on the proliferative response of B cells to anti-μ. In contrast to mitogen-induced T-cell proliferation, where CY A must be added during the first 24 hr of culture for inhibition to be observed, even after 48 hr the addition of CY A caused substantial inhibition of anti-μ stimulation.

Klaus & Hawrylowicz (42) extended these studies of the effects of CY A on B-cell activation by using the two-stage culture system originally described by DeFranco et al (43). Although CBA/N mice fail to generate an antibody response or to synthesize DNA in response to anti-μ, anti-Ig does activate CBA/N cells to enter the G_1 phase of the cell cycle. This activation can then be simply assayed by stimulating the cells with LPS, which rapidly induces DNA synthesis. In a similar manner, the tumor-promotor PMA functions to prime B cells to respond to LPS. When either normal or defective B cells were first stimulated by anti-μ and then by LPS, CY A inhibited activation by blocking an event that occurred within 4 hr of initial stimulation. CY A prevented cells from entering G_1, but once a B cell had passed this step the response was CY A resistant. Surprisingly, as noted above, continuous stimulation of normal B cells with anti-μ was CY A susceptible throughout G_1 and probably into S. Unlike anti-Ig, PMA causes only a portion of B cells to enter G_1, but this PMA-induced primary step was totally CY A resistant. One way to resolve these findings is to postulate that B cells require two signals for activation. Signal 1 drives a

resting (G_0) cell into G_1, and signal 2 drives it from G_1 into S. B cells can receive two biochemically distinct forms of signal 1 : Anti Ig–induced signal 1 is CY A sensitive while PMA-induced signal 1 is CY A resistant. Both PMA and LPS bypass the CY A–sensitive step and short-circuit the physiological activation of B cells through the Ig receptor. However, because CY A completely inhibits the proliferative response induced by the continuous presence of anti-Ig, it is necessary to postulate that it must also block the anti-Ig-induced signal 2. Alternatively, while anti-Ig may directly drive B cells into G_1 and this step is inhibited by a direct effect of CY A on the responsive B cells, the sensitivity of anti-μ-induced proliferation to inhibition by CY A may be secondary to inhibition of B cell growth factor (BCGF) production by residual T cells in the B-cell cultures.

Lillehoj et al (13) confirmed that CY A had no effect on the B-cell proliferative response to LPS and also demonstrated that CY A had no effect on the expression of IL-2 or transferrin receptors as assayed on the FACS with rat monoclonal antibodies. IL-2 receptor expression was not directly evaluated on anti-μ-stimulated B cells in these experiments.

Studies on Human Lymphocytes

The effects of CY A on human B-lymphocyte activation by various stimuli are quite similar to those seen with mouse B lymphocytes. There appear to be both sensitive and resistant components of the B lymphocyte–activation process. Muraguchi et al (44) demonstrated that CY A could completely inhibit B-cell proliferative responses to anti-μ and BCGF, while it also had considerably less potent suppressive effects on the response induced by *Staphylococcus Aureus* Cowan I (SAC). The effect on the anti-μ response appeared to be selective for the activation phase of the response. Thus, CY A was required during the first 24 hr of culture and inhibited RNA synthesis by anti-μ-stimulated B cells in the presence or absence of BCGF. Although low concentrations of anti-μ can stimulate RNA synthesis in small B cells, DNA synthesis requires the presence of BCGF. These data suggest that CY A inhibits DNA synthesis of small B cells in response to anti-μ plus BCGF, because the drug inhibits the mechanism by which small B cells can be induced to acquire responsiveness to BCGF. Addition of CY A to cultures that had been preactivated with anti-μ for 30 hr did not suppress the response to BCGF.

Tosato et al (45) demonstrated that CY A consistently suppressed the activation of human B lymphocytes into Ig-secreting cells when stimulated with the T-dependent activator, pokeweed mitogen (PWM). In contrast, CY A had no effect on Ig production in cultures stimulated with the T- and monocyte-independent activator, the B95-8 strain of Epstein Barr Virus

(EBV). One additional aspect of these studies is worthy of mention and relates to the effects of CY A on the activation of suppressor T cells for Ig production. It is known that EBV-seropositive individuals have T cells in the peripheral blood that suppress the activation of autologous B cells by EBV, whereas EBV nonimmune individuals lack such suppressor T cells. CY A prevented activation of such suppressor cells because CY A containing cultures of EBV-immune, autologous B, and T lymphocytes produced very high levels of Ig-secreting cells; and no inhibition of Ig production was observed. In contrast, during the course of EBV-induced infectious mononucleosis, suppressor T cells become activated in vivo, and the addition of such T cells to normal lymphocytes results in marked inhibition of Ig production of the B cells, which is totally unaffected by the presence of CY A. Thus, the activation of suppressor T cells is CY A sensitive, but once fully activated the suppressor cells appear to be resistant to the immunosuppressive effects of CY A. The use of CY A to prevent allograft rejection has been associated with a rise of antibody titers to EBV and to the appearance of a small number of EBV-containing lymphomas. It is thus possible that the impairment of EBV-specific T-suppressor function by CY A may be of significant clinical relevance.

IS THERE A CY A RECEPTOR?

The identification of a specific cell-surface or cytoplasmic receptor for CY A would greatly facilitate a detailed examination of the biochemical basis for its mechanism of action. A number of groups have attempted to address this problem, but, as will be apparent from the discussion to follow, no clear picture emerges from the heterogeneous nature of the experimental findings. At the outset it is worth reemphasizing that some of the difficulties encountered are obviously secondary to the hydrophobic nature of the CY A molecule.

Ryffel et al (46) originally described the specific binding of ^{3}H-CY C to mouse lymphocytes. This compound has a very similar spectrum of activity compared to CY A, and at the time these studies were performed it was the only available compound with a high specific activity. The binding of ^{3}H-CY C was specific, saturable, and reversible. Furthermore, the radioactive ligand was displaceable by CY A as well as by CY C. ^{3}H-CY C bound with a high affinity (K_D 1.5×10^{-7}M) to a single class of receptors. Although both B and T lymphocytes bound the ligand, the binding capacity for T lymphocytes was higher than that observed for B lymphocytes; thymocytes had a higher binding capacity than peripheral T lymphocytes. The binding of ^{3}H-CY to human cells was also studied by Ryffel and coworkers (47).

Specific ^{3}H-CY C binding to lymphocytes reached a plateau within 30 min at 37°C and was rapidly reversible with a half-life of 3 min. The radiolabeled CY C could be found in the plasma membrane. Nylon wool–separated cells did not differ either in affinity or capacity for ^{3}H-CY C binding from whole or monocyte-depleted lymphocytes. In fact, polymorphonuclear leukocytes showed similar ^{3}H-CY C binding characteristics and about the same number of sites/cell. Thus, the binding characteristics of ^{3}H-CY C for lymphocytes and other blood cells clearly do not predict the biological effect of this drug on different cell populations.

Palacios (48) approached the problem of the CY A receptor from a different point of view. He observed that the CY A strongly suppressed the proliferation of human T cells stimulated with monoclonal antibody OKT3, but when CY A was added to the cultures 72 hr after initiation, the drug no longer suppressed. These experiments raised the possibility that CY A and OKT3 compete for the same binding site. In experiments designed to test this hypothesis CY A significantly inhibited the binding of OKT3 but not of OKT4 or OKT8 monoclonal antibodies, and pretreatment of target cells with CY A significantly decreased cytotoxicity mediated by OKT3 and C but not that mediated by OKT4 and C. These results raise the possibility that CY A interferes with T-cell activation by binding to one of the critical cell-surface structures involved in most but perhaps not all processes of T-cell activation. Since OKT3 is not found on B lymphocytes and since CY A does directly affect B- cell activation by certain stimuli, one must of course raise the issue of the nature of the CY A receptor on the B lymphocyte. Ryffel et al (47) have attempted to extend Palacios's findings by showing that the specific binding of ^{3}H-CY C could be reduced by pretreatment with either Con A, PHA, or OKT3. However, since the simultaneous additions of the mitogens at high concentrations did not inhibit ^{3}H-CY C binding, these investigators felt that the mitogen receptor and CY A receptor were not identical but were closely associated on the cell membrane so that the interactions of the mitogens with their receptors induced a rearrangement of membrane proteins including the CY A receptor. This concept does not fully explain Palacios's observations unless one postulates that pretreatment with CY A specifically alters the state of the OKT3 antigen so that its interaction with antibody is defective.

Legrue et al (49) have recently performed an extensive series of studies that have raised a number of questions about the significance of the previous CY A–binding studies. These investigators used ^{3}H-CY A and found that there was no difference in the in vitro uptake of this ligand by B or T cells. Surprisingly, even human erythrocytes bound substantial amounts of ^{3}H-CY A at very low drug concentrations. All the cell types

examined achieved maximum uptake of the drug by 2 min, and Scatchard analysis demonstrated the presence of two ^{3}H-CY A association constants. The low-affinity site had a K_D of 2.8×10^{-7}M, and the high-affinity site had a K_D of 6.3×10^{-9}M. B lymphocytes displayed two binding constants of K_D 8×10^{-7}M and 2×10^{-9}M, whereas T lymphocytes bore a single low-affinity site (K_D 3.4×10^{-7}M). Cultured human kidney cells had a Scatchard plot similar to that of T cells (1.7×10^{-7}M).

To test the possibility that the binding studies performed with CY A simply measured partition of the drug into the lipid bilayer, liposomes of defined chemical composition were prepared using a 1:1 mixture of egg yolk phosphatidylcholine and cholesterol. Pure lipid liposomes bound ^{3}H-CY A with a K_D of 2×10^{-8}M. Of the ^{3}H-CY A associated with the liposomes 95% could be displaced by incubation for 30 min in a 500-fold excess of unlabeled CY A. Surprisingly, there was no difference in the binding of ^{3}H-CY A to pure lipid liposomes or to liposomes prepared using lipids extracted from peripheral blood lymphocytes. Taken together, the studies of Legrue et al (49) argue against a specific CY A receptor. Furthermore, these studies raise the possibility that CY A might function in the plasma membrane in a manner similar to that of lipid soluble anesthetics by increasing lipid fluidity and uncoupling electrochemical action potentials. Thus, the specificity of CY A in the modulation of immune reactivity might arise from differences in the electrochemical mechanisms used during the activation of lymphocyte subsets and not from differences in the specific binding or net uptake of the drug.

The issue of CY A receptors has been further complicated by the recent studies of Merker & Handschumacher (50), who have raised the possibility that CY A may be specifically localized intracellularly. They performed a series of uptake studies on a subline of the BW5147 thymoma that was found to be very sensitive to growth inhibition by CY A. Of the drug concentrated by the cells 70–80% was found in the cytosol (i.e. the 100,000-*g* supernatant) of lysed cells. A complex between drug and a 15,000–20,000 M_r macromolecule was identified. This complex could also be formed by adding the drug to cytosol preparations prepared from non–drug exposed cells. No estimate of the binding affinity was made in these studies. A similar component could be isolated from a subline of BW5147 that was resistant to growth inhibition by CY A. At present it is difficult to postulate a relationship between the cytosolic-binding component and the mechanism of action of CY A, particularly because there is little apparent relationship between the growth inhibition properties of CY A and its immunosuppressive activity. One intriguing possibility is that this component has a natural ligand that plays a role in T-cell activation and that CY A functions primarily by displacing this ligand from the binding component.

CY A AS A THERAPEUTIC AGENT FOR AUTOIMMUNE DISEASE

Studies in Experimental Animals

The ability of CY A to inhibit T-cell activation in vitro strongly suggests that this drug would have the potential to function as a potent immunotherapeutic agent in autoimmune disease. The early studies of Borel et al (3) described impaired delayed and impaired contact sensitivity in CY A–treated animals. Thomson et al (51, 52) extensively analyzed the effects of CY A on the mechanisms underlying the generation of delayed-hypersensitivity reactions in both mice and guinea pigs. Daily oral administration of CY A markedly suppressed delayed sensitivity responses to ovalbumin in the guinea pig when given throughout the interval between immunization and skin testing on day 14. Profound suppression of the skin test was also seen when CY A was given only immediately prior to skin testing. These observations are consistent with the view that CY A inhibits the lymphokine production needed both for T-cell proliferation and for the recruitment and activation of macrophages. One surprising finding in these studies was that the administration of CY A only on days 0–4 caused augmentation of subsequently evoked skin reactivity to ovalbumin. This result raises the possibility that withdrawal of CY A in the presence of a depot of antigen in adjuvant may have allowed an early inhibitory effect on suppressor T cells to become manifest.

CY A has been shown to be highly effective in the prevention of a number of experimental autoimmune diseases induced by the administration of antigen in adjuvant. EAE (experimental allergic encephalomyelitis) has been successfully prevented in rat, guinea pig, and rhesus monkey for as long as the drug was administered (3, 53). However, once treatment was stopped clinical signs occurred in most animals although they developed milder symptoms than controls. Delayed or therapeutic treatment of EAE also resulted in impressive improvement in both rats and guinea pigs. It is likely that CY A prevents EAE by inhibiting the expansion of antigen-reactive cells and their production of mediators, but when the drug treatment is terminated and antigen presists in the host there is a prompt resumption of activation of effector–T cell function. The effects of CY A are clearly different from the effects of cytotoxic agents like cyclophosphamide in which case the disease does not recur.

EAE can also be adoptively transferred to normal hosts following stimulation of the donor T cells in vitro with antigen or mitogen. Oral treatment of recipients prevented the development of adoptive EAE following transfer of in vitro–stimulated lymphoid cells (54). Cells obtained from sensitized donors that had been treated with CY A were resistant to in

vitro expansion and failed to transfer EAE. When CY A was added to the in vitro expansion cultures at the same time as either antigen or mitogen, the spleen cells were prevented from transferring clinical signs of EAE. Hinrichs et al (55) also demonstrated that when effector cells were derived from animals that had received CY A after a delay of 2–4 days, clinical EAE was readily induced in recipients. This result demonstrates that CY A can inhibit effector-cell development in vivo but that antigen-sensitive cells that are developmentally arrested by CY A in vivo can develop to an effector-cell stage in vitro when restimulated by antigen in the absence of CY A.

Nussenblatt et al (56, 57) have reported the successful inhibition of S antigen–induced experimental autoimmune uveitis in Lewis rats with CY A. CY A was capable of totally preventing the clinical appearance of disease even when administered on an every other day schedule or when begun several days after immunization. Treated animals had normal levels of antibody to the S-antigen, but their in vitro proliferative responses to S-antigen were greatly diminished. Rats that were treated seven days after antigen administration had a shift in their histopathology to a more granulomatous type of disease.

CY A has also produced marked therapeutic effects in diabetes in the BB rat, a strain in which insulin-dependent, nonobese, ketotic diabetes mellitus develops spontaneously. Histologically, in affected animals there is intense peri-islet invasion by mononuclear cells with resultant complete destruction of β cells within 1–3 weeks of the appearance of the glycosuria. Diabetes in this rat strain seems to resemble type-1 insulin-dependent diabetes in man. None of the CY A treated BB rats developed glycosuria, and their pancreases were histologically normal (58). When CY A was discontinued in the BB rat at 120 days of age, diabetes occurred in less than 25% of the animals.

Diseases Aggravated by CY A

Although CY A has also been used successfully to treat experimental thyroiditis in rats (59) and to inhibit the experimental production of autoantibodies in mice (60), there are several well-described autoimmune disease models in which when CY A has either failed to cure or has in fact aggravated the disease. Examination of these studies is instructive and should offer further insight into the basic mechanisms of action of CY A. During the first few weeks after hatching, the obese strain (OS) of chickens show a spontaneous autoimmune thyroiditis (SAT) mimicking Hashimoto's thyroiditis in man in all clinical, histopathological, and immunological aspects. G. Wick et al (61) developed a model in chickens in which CY A was able to prolong skin graft survival, but the same dose of CY A did not prevent the development of SAT in OS chickens or improve already

established thyroiditis. Furthermore, when CY A was given to OS embryos, it resulted in more severe disease and higher titers of antithyroglobulin antibody. These results strongly favor the view that CY A may augment this disease by preventing the development of T suppressor cells.

The inhibition of suppressor T cell activity can also be postulated to explain the very unusual findings of Glazier et al (62). These investigators observed that lethally irradiated rats treated with CY A developed classic GVHD when reconstituted with either syngeneic or allogeneic bone marrow following CY A withdrawal. Syngeneic GVHD could be adoptively transferred to irradiated but not to normal syngeneic recipients. Furthermore, normal spleen cells failed to prevent the development or adoptive transfer of syngeneic GVDH when given at the time of marrow transplantation to CY A–treated syngeneic chimeras. Although a clear explanation for these rather enigmatic findings cannot be easily drawn from the experimental data, it would appear that some adoptive response is required from the recipient of even syngeneic bone marrow in this model and that certain aspects of this response—perhaps the development of suppressor-T cells that inhibit anti-self effectors—are inhibited by CY A.

It has been unequivocally demonstrated that in several of the models of autoimmune disease described above the disease produced by antigen administered in adjuvant can be totally suppressed by CY A when it is given during the induction phase. However, some of the complexities of the experimental use of CY A even in these models have been pointed out by the studies of Kaibara et al (63), who investigated the effects of CY A on collagen arthritis in rats. CY A readily prevented the development of arthritis in type-II collagen-immunized rats when the agent was given prophylactically; a 7-day course of CY A was nearly as effective as a 14-day course. However, when CY A was administered only during the immediate preclinical phase of arthritis, CY A–treated rats showed a marked enhancement of the disease in a dose-dependent manner with enhanced delayed skin reactions and suppressed antibody responses. CY A also showed an enhancing effect on established disease, and this enhancement was accompanied by an augmentation of skin reactions. These results must be contrasted with the findings in the uveitis model (56, 57) where CY A given seven days after immunization efficiently prevented disease. The most likely explanation for these findings is that the administration of CY A late in the course of immunization inhibited the clonal expansion of T-suppressor cells while permitting the expansion of helper T cells that produce severe arthritis. The studies of Tosato et al (45) described earlier clearly showed that the activation of certain populations of suppressor-T cells, at least in vitro, can be prevented by CY A.

Clinical Use in Man

In spite of the possible theoretical reservations raised by the above studies in experimental animals, CY A has already been used successfully in several human disease states. Nussenblatt et al (64, 65) directly applied what they had learned from the rat model of experimental uveitis and treated a number of patients with posterior uveitis whose lymphocytes responded in vitro to S-antigen. In most patients there was a rapid response to CY A with improvement in visual acuity and a diminution in ocular inflammatory activity. Curiously, the one treated patient whose lymphocytes did not proliferate in response to S-antigen did not respond to CY A therapy. In CY A–responsive patients, in vitro lymphocyte responses were not abrogated during CY A therapy. These investigators postulate that CY A inhibits disease activity by inhibiting the recruitment of immunoreactive cells needed for the continued amplification of the disease process even in a subject where the inflammatory response is well established prior to therapy.

The second animal model to be extended to man is the use of CY A to treat type-I diabetes mellitus. Although the loss of β-cell mass at the time of clinical expression of the disease is probably substantial, many patients undergo a partial recovery and temporary clinical remission. This phase lasts for a few weeks or months and is followed inevitably by increasing insulin requirements and lability of metabolic control. When 30 patients were treated with CY A within six weeks of diagnosis, 16 became insulin independent, while only 2/11 patients who entered the study 8–44 weeks after diagnosis achieved this state (66). The patients who did not require insulin were still carbohydrate intolerant, and it is still unknown whether complete relapse will occur in most patients when CY A administration is discontinued. Nevertheless, these very preliminary results are an impressive beginning and further substantiate the accumulating evidence that type-I diabetes mellitus is a disease with an immunologic basis.

Although the results in uveitis and diabetes are impressive, it has not proven simple to transfer some of the black and white results of the animal models to the clinical situation. For example, CY A will readily prevent acute GVHD in rats lethally irradiated or treated with cyclosposphamide and then reconstituted with allogeneic bone marrow (67). Acute GVHD does not appear after cessation of the drug, although chronic GVHD may be seen. Tutschka et al (68) extended these studies to humans and used CY A as the sole immunosuppressive agent in 22 patients who received allogeneic bone marrow transplantation as therapy for aplastic anemia and hematologic malignancies. Although engraftment was not impaired, CY A did not produce a striking decrease in the incidence or severity of acute

GVHD. Furthermore, although CY A markedly accelerated the recovery of immunocompetence in the rat model, it did not appear to do so in man. The incidence of interstitial pneumonia, the major clinical manifestation of the suppressed state, was identical in CY A–treated and nontreated subjects.

SUMMARY AND CONCLUSIONS

Over the past ten years a great deal of phenomenology has accumulated about the mechanism of action of CY A on the immune response. Although considerable progress has been made in both cellular and molecular immunology, we still cannot say with any degree of confidence that we understand which step in the activation of lymphocytes is blocked by CY A. Our inability to make rapid progress reflects both our lack of knowledge and the lack of appropriate methods to assay the earliest events in lymphocyte activation that seem to be affected by CY A. In summary, a few points are worthy of emphasis. First, CY A is clearly not a T cell–selective agent, as a number of studies have demonstrated direct effects on B-cell activation by certain stimuli. Second, there appears to be an alternative pathway of lymphocyte activation that is CY A resistant. The best examples are the activation of B lymphocytes by LPS or EBV and also perhaps the activation of T lymphocytes by PMA. Third, although CY A does not inhibit the effector function of macrophages, more careful studies should be performed to rule out the effects of CY A on the function of macrophages and other types of accessory cells in the afferent limb of the immune response. Lastly, as new methods become available to quantitate each step in the process of lymphocyte activation, the effects of CY A on the assay should be measured. Studies over the past year have shown that CY A fails to block the mitogen-induced increase in $[Ca^{+2}]i$ but does readily inhibit the transcription of IL-2 mRNA. We are thus obviously narrowing the gap in our search for the critical CY A–sensitive step.

The clinical use of CY A to prevent allograft rejection has been aptly described by Kahan (69) as an example of the presence of saints Cosmas and Damian in the twentieth century. These remarkable internist-surgeon twin brothers performed what is called the miracle of the black leg: In the fifth century AD they successfully transplanted the leg of a recently deceased Ethiopian moor to the stump of an aged sacristan of the church. The story is even more remarkable in that both brothers were beheaded in 287 AD and the famous operation took place at one of their reappearances. Unfortunately, some of the enthusiasm for the use of CY A both for the transplant recipient and in autoimmune disease has been dampened by the presence of significant nephrotoxicity (70). Although lower-dose protocols may reduce the incidence of this serious side effect, it remains a significant

problem. One area of future study that should be exploited would be to use CY A to generate clones and lines of antigen-specific T suppressor cells which might then be administered to the transplant recipient. Perhaps during the last 15 years of the twentieth century we will be able to duplicate the miracle Cosmas and Damian performed in the fifth century—the induction of donor-specific unresponsiveness without the administration of immunosuppressive drugs.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 425–53

THE COMING OF AGE OF THE IMMUNOGLOBULIN J CHAIN

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INTRODUCTION

The existence of a third immunoglobulin polypeptide, the J chain, is often overlooked. The oversight stems in part from the differences between the J chain and the other immunoglobulin polypeptides. Unlike the heavy and light chains, the J chain is incorporated only in the polymeric immunoglobulins, IgM and IgA (1, 2). Unlike the heavy and light chains, the J chain does not contribute to antibody specificity; as an Fc-linked component (3, 4), it participates only in polymer-specific effector functions such as binding to the transport receptors on epithelial cells (5, 6). Moreover, unlike the heavy and light chains, the J polypeptide does not belong to a superfamily of structurally related molecules (7), and thus it has presumably evolved independently.

The oversight of the J chain also stems from uncertainties concerning its structure and biosynthetic function(s). Until recently the characterization of J-chain structure was at a rudimentary level. Sequence data were available only for the human protein (7), and with such limited information it was not possible to identify conserved features or to deduce structure-function relationships. Characterization of the biological properties of the J chain was more advanced, but the role of the protein in antibody biosynthesis remained unclear. On the one hand, studies of polymer assembly (8–10) indicated that the primary function of the J chain is to initiate the polymerization of IgM and IgA. On the other hand, the synthesis of J chain by IgG-secreting cells (11, 12) and by some pre-B lines (13, 14) raised the possibility that J chain performs some general function in the immune response.

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In the decade since the last comprehensive reviews of the J chain (15, 16), considerable progress has been made in defining its structure and function. The purpose of this communication is to evaluate the new evidence, indicating both the questions that have been answered and those that remain to be resolved. Hopefully, such an examination will serve to emphasize the key roles that the immunoglobulin J. chain plays in antibody biosynthesis.

STRUCTURE OF THE J CHAIN

Primary Structure

Early attempts to determine the primary structure of the J chain were hampered in several ways. It was difficult to obtain enough protein for analysis because the J chain comprises a minor portion of the polymer structure and, in species other than the human, sources of polymer-starting material were limited. In addition, the J chain is particularly susceptible to proteolytic attack (17) so that the introduction of trace amounts of enzyme during isolation and storage often caused extensive degradation. Finally, the structure of the J protein itself presented problems: The high content of lysine, arginine, and cysteine residues made it difficult to isolate peptides suitable for analysis (18).

The development of recombinant DNA technology and more sensitive sequencing methods provided the means to overcome such difficulties. The primary structure of the murine J chain was the first to be derived by use of these methods. J chain–specific clones were isolated from libraries of plasmacytoma cDNA, and the nucleotide sequence of inserts containing coding information was determined (19). The amino-acid sequence deduced from these data was then verified by direct determinations of the amino- and carboxy-terminal residues of the mature protein and by sequence analysis of selected peptides (20). More recently, the entire sequence of the human J chain has been inferred from nucleotide analyses of cloned genomic DNA (21). The results of these studies are summarized in Figure 1.

The data show that the J chain is a larger protein than expected from earlier studies (7). Both the murine and human chains were found to contain a total of 137 residues; in the human polypeptide an additional residue—a cyclized glutamic acid—is present at the amino-terminus, and an internal proline has been deleted. Other aspects of the sequences shown in Figure 1, however, support the findings of previous biochemical investigations (15, 16). Thus, both chains have a relatively large number of cysteine residues scattered throughout the sequence. Both chains are rich in acidic amino acids, consistent with their rapid anodal migration in alkaline-urea polyacrylamide-gel electrophoresis and also with isoelectric pH values of

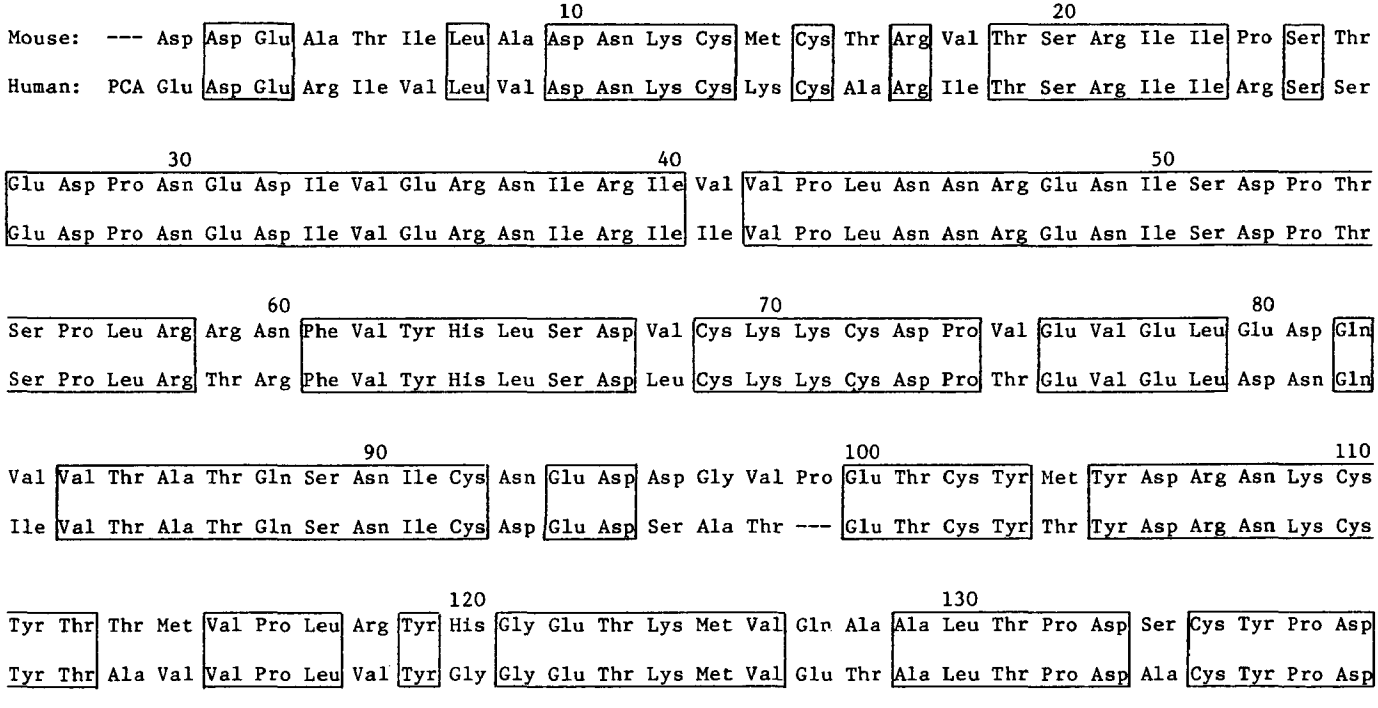

Figure 1 Comparison of the amino-acid sequences determined for mouse and human J chains (7, 19, 21). Boxes indicate regions or residues of identity. Dashed lines indicate gaps introduced to maximize the homology.

approximately four. Both chains have abnormally low contents of glycine, serine, and phenylalanine, and they lack tryptophan. The combination of these characteristics is responsible for the structural uniqueness of the J chain; no significant homology has been observed with any other proteins including the immunoglobulin-heavy and -light chains (7).

The most striking feature of the sequences in Figure 1 is their similarity. Of the 137 residues in mouse and human J chains, 106 or 77% are identical. The region encompassing positions 27 to 58 is particularly conserved with only one valine-isoleucine replacement in 32 residues. Moreover, features important to the three-dimensional structure have been maintained. Thus, both the mouse and human J chains contain eight cysteine residues that occupy identical positions, and both proteins exhibit stretches of hydrophobic residues that alternate in the sequence with stretches of predominantly hydrophilic character. The homology between the mouse and human J chains is comparable to that observed for the regions of IgM and IgA monomers involved in the polymerization process (19). These include the carboxy-terminal constant domains and the adjoining secreted termini that contain the penultimate cysteine residue through which polymerization is effected (3, 4). It can be seen from the data compiled in Table 1 that the $C_\mu 4$ and $C_\alpha 3$ domains of the mouse are 78% homologous with the human $C_\mu 4$ and $C_\alpha 3$ (22, 23), a value very similar to that obtained for the J chains; the homologies of the respective μ- and α-secreted termini fall within the same range. In contrast, the sequences of other C_μ and C_α domains and the sequences of all the C_γ domains (24, 25), including the carboxy-terminal, have diverged to a significantly greater extent. These comparisons indicate that the structural requirements for polymerization have imposed such selective constraints that the J chain and the carboxy-terminal regions of the μ and α chains are the most conserved elements in the immunoglobulin system.

The question as to why the polymerization process has been maintained over the evolution of the immunoglobulins remains to be answered. Although pentamer IgM was the first secreted antibody to appear in the primitive vertebrates, mechanisms for producing monomeric antibodies with higher antigen affinity and specificity have subsequently evolved. One possible explanation for the conservation is the advantage conferred by multivalence. Because the polymeric antibodies can more effectively complex pathogens displaying repeating sets of determinants on their surface, they may play an essential role in protection against disease—pentamer IgM by acting in the early stages of infection, and polymeric IgA by protecting the body surfaces. A second possible explanation is that the polymerization of IgM is an obligatory event in the differentiation of the antibody-producing cell. Some component of the polymerizing system,

Table 1 Homology of the J chain domain and the heavy-chain constant region domains from man and mouse

Comparison of human vs mouse	% Homology in domain				
	1	2	3	4	COOH terminus[b]
J-chain domain[a]	77 (137/137)				
μ-chain constant domain	48 (106/106)	59 (106/106)	53 (107/107)	78 (111/111)	89 (19/19)
α-chain constant domain	42 (98/98)	64 (102/102)	78 (77/77)		68 (19/19)
γ-chain constant domain	62 (48/92)	66 (110/110)	57 (103/103)		

[a] In each domain column the ratio in the parenthesis shows the number of amino acids compared in the homology determination relative to the total number of amino acids in that homology unit. The percentage value is the percent homology that was calculated by determining the number of sequence identities among the amino acids compared. Sequence gaps (insertions or deletions) were not counted in the homology calculations.

[b] COOH terminus, carboxyl-terminal sequences of secreted μ and α chains.

[c] Human $C_{\gamma 1}$ vs mouse $C_{\gamma 2a}$; comparisons of the homology between human $C_{\gamma 4}$ and mouse $C_{\gamma 3}$, human $C_{\gamma 2}$ and mouse $C_{\gamma 1}$, and human $C_{\gamma 3}$ and mouse $C_{\gamma 2b}$ gave similar results.

either a protein or RNA product, may be required for the switch in immunoglobulin class that normally follows the pentamer IgM response.

Three-dimensional Structure of the J Chain

In contrast to the progress made in resolving the primary structure of the J chain, little direct evidence has been obtained about the three-dimensional structure. It has not been possible to determine the native conformation by studying the isolated J chain because the reducing conditions required to free J chain from immunoglobulin polymers also reduce all the intra–J chain disulfide bonds (20). Nor has it yet been possible to apply an alternative approach, namely, X-ray crystallographic analysis of the intact polymers or their Fc portions.

In view of these experimental limitations, several attempts have been made to deduce the folding of J chain from its amino-acid sequence. Cann et

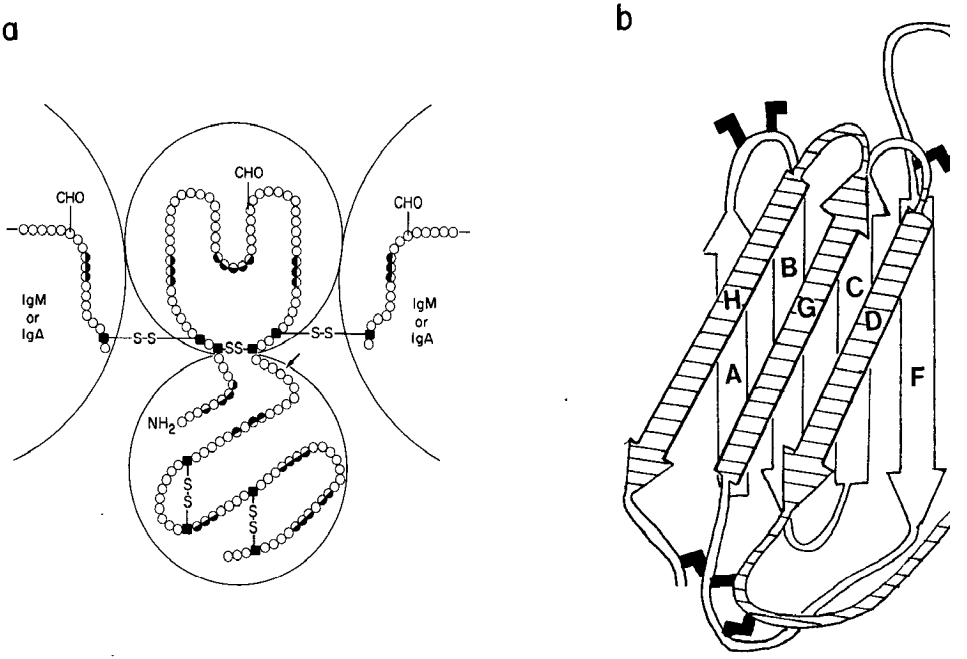

Figure 2 Hypothetical models for the three-dimensional structure of the J chain. (*a*) Schematic drawing of a two-domain model in which half closed circles indicate stretches of hydrophobic amino acid residues, closed boxes denote half-cystine residues, and open circles represent all the remaining amino acid residues. The arrow indicates a peptide bond hypersensitive to cleavage by subtilisin. (*b*) Schematic drawing of a one-domain model based on the folding topology of superoxide dismutase. The arrows indicate β strands, and the thick zigzag lines denote one of the possible arrangements of disulfide bridges. In this scheme, the two N-proximal half-cystines are postulated to form interchain disulfide bonds to Ig chains, and the end of β strand D is thought to represent the site of proteolytic attack.

al (19) have proposed a two-domain structure based on the sequence differences between the amino-terminal and carboxy-terminal halves of the J polypeptide (Figure 2a). The amino-terminal half was postulated to form a symmetrically arranged domain that contains a single internal disulfide bridge and two free -SH groups available for intermonomer linkage. The carboxy-terminal sequence, on the other hand, was postulated to generate a second domain with a more irregular structure and two short disulfide bridges. This model is consistent with predictions of the Chou & Fasman algorithms (26) that the amino-terminal sequence has a high propensity for forming β-sheets, whereas the carboxy-terminal sequence displays a mixture of α-helix and β-sheet propensities. A two-domain model is also

consistent with data on J-chain proteolysis (17). Limited exposure of pentamer IgM to subtilisin has been shown to degrade the carboxy-terminal portion of the J polypeptide selectively without appreciably altering the structure or effector functions of the parent polymer (27).

Recently, Zikan et al (28) have used a newly developed computer program (29) to predict the secondary structure of the J chain. The program generates a series of amino acid sequence profiles describing properties important to folding (hydrophobicity, electric charge, secondary structure propensities) and allows for cumulative averaging of properties from homologous sequences. The cumulative profiles obtained for human and mouse J chain (Figure 3) strongly suggest that the protein consists of eight antiparallel β-strands organized into two antiparallel sheets. A sketch of one possible arrangement is given in Figure 2b. The prediction of a single β-barrel domain was based on the similarity of the J-chain sequence profiles to those obtained for known antiparallel β-barrel structures such as the immunoglobulin V_L domains (30) and superoxide dismutase (31). In each case, the profiles showed a striking correspondence between peaks of hydrophobicity and β-sheet propensity and minima of reverse turn propensities. The β-barrel model is consistent with circular dichroism measurements of isolated, reoxidized J chain (29); the renatured protein was found to have a β-sheet content of 34%, in good agreement with the predicted value of 37%. Moreover, the model is consistent with the susceptibility of the J-chain disulfide bonds to reduction (20, 26). The prediction that all the cysteine residues are located at the exposed loops between β-strands would allow the S-S bonds to be cleaved by mild reduction in aqueous solution. The most attractive feature of the model, however, is the homology between the postulated J-chain domain and the domains of the other immunoglobulin chains. The possibility that an immunoglobulin-like fold can be generated from an unrelated amino-acid sequence would explain the ability of the J chain to interact with the $C_\mu 4$ and $C_\alpha 3$ domains and perhaps also explain the conservation of the J-chain primary structure.

It should be emphasized that the models shown in Figure 2 represent guesses—albeit educated ones—of the J-chain conformation. Because methods for computing secondary structure depend in large part on statistical analyses of amino-acid sequences, the predictions have not proved to be highly accurate. Resolution of the three-dimensional structure of the J chain must, therefore, await X-ray crystallographic analyses of the parent polymers. Until such studies are accomplished, the proposed models can provide frameworks for designing experiments to probe the structure-function relationships of the J chain.

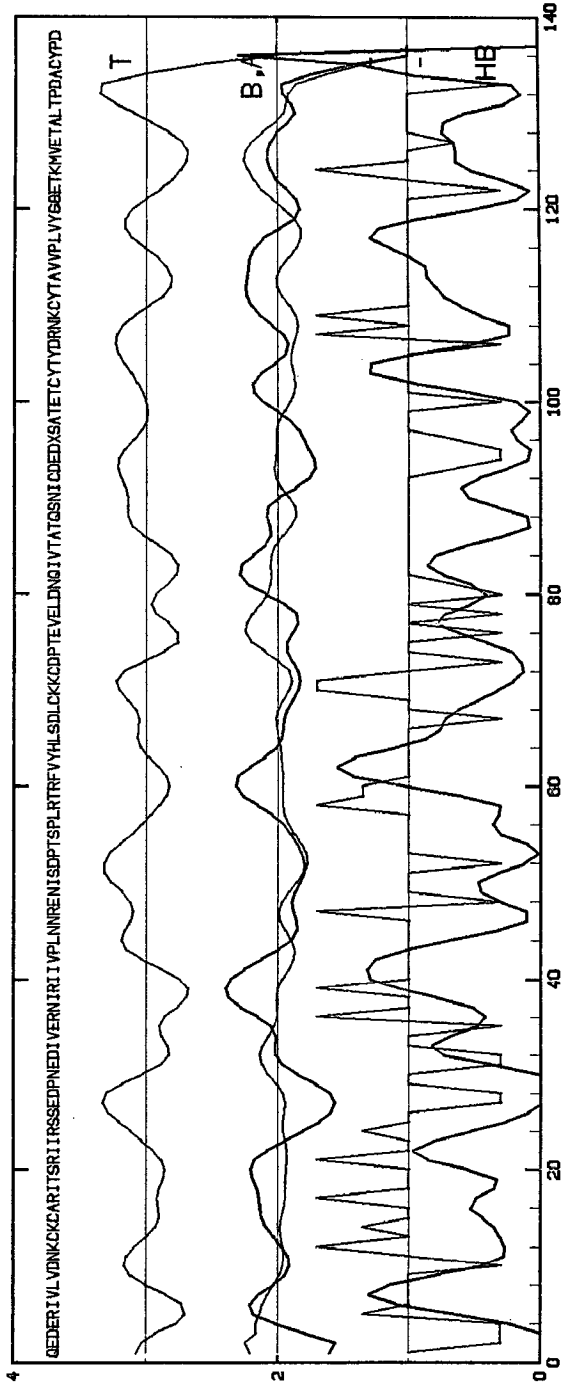

Figure 3 Amino acid–sequence cumulative profiles of the J chain computed from the data for the human and mouse polypeptides. From top to bottom: amino acid sequence of the human J chain; reverse turn propensity (T); α helix (thin line) and β-sheet (heavy line) propensities (B,A); profile of charge residues (+ −); and hydrophobicity (HB). Short lines below the box indicate deduced β strands.

BIOSYNTHESIS OF THE J CHAIN

An understanding of the function(s) of the J chain requires knowledge not only of its protein structure, but also of its biosynthesis. Through the use of the newly developed tools of molecular biology, many of the processes involved in J-chain expression have been delineated. These include transcriptional and processing events in the nucleus as well as translational and posttranslational events in the cytoplasm.

Gene Structure and Regulation

In the mouse, the J chain is encoded by a single gene that is located on chromosome 5 (32) and thus is not linked to the immunoglobulin heavy- and light-chain genes. Sequence analyses of genomic DNA (33) have shown that the J-chain coding information is contained in 4 exons (Figure 4): the first specifies the 5′ untranslated and leader sequences; the second and third encode the amino-terminal half of the J protein, residues 1–40, and 41–67, respectively; and the fourth encodes the entire carboxy-terminal half of the J chain and a large 3′ untranslated sequence. The exons are separated by successive introns of 1.5, 3.5, and 1 kb so that the information for the 23–amino acid leader peptide and the 137–amino acid mature protein spans more than 7 kb of genomic DNA. This structure is independent of J chain expression. No differences have been detected in the DNA from J chain–expressing and J chain–nonexpressing cells by either restriction mapping or partial sequencing (33, 34). Thus, the J-chain gene, unlike the other immunoglobulin genes, does not require translocation of DNA segments for transcription.

The exon organization of the J chain differs from that of the heavy- and light-chain genes in several other aspects. With the exception of the leader sequence, the J-chain exons do not appear to correspond to functional

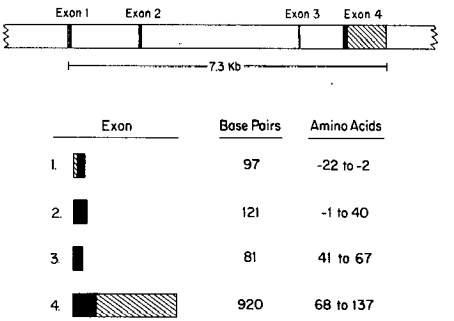

Figure 4 Schematic diagram of the exon-intron organization of the murine J-chain gene.

domains in the protein. There is a rough correlation between the exon contents and the amino-acid sequence in that exons 2 and 3 specify the so-called mirror image segments (19) of the amino-terminal half of the molecule and exon 4 specifies the carboxy-terminal half. However, this organization is clearly distinct from that of the heavy- and light-chain genes where the exons encode entire variable or constant region domains. The exon-intron boundaries also have different characteristics. In the J-chain gene, the noncoding sequences are inserted at various positions in the exon codons, whereas in the heavy- and light-chain genes the noncoding sequences invariably interrupt the exon codons after the first base pair (35). These differences in exon structure provide further evidence that the J chain and heavy and light chains have evolved independently.

The sequences upstream of the J-chain gene, on the other hand, are organized similarly to the upstream regions of immunoglobulin V genes (Figure 5). The common features include the two transcription initiation sites—one located 32 bp and the other 26 bp 5′ to the start of the coding information—and a short TATA sequence located approximately 25 bp further upstream (36, 37). The most striking common features, however, are the two tissue-specific elements, the decanucleotide sequence that starts at position −74 and the pentadecanucleotide that starts at position −136. Similar elements have been found upstream of all human V_κ and mouse V_κ genes examined to date, as well as upstream of mouse V_λ genes (38, 39). Moreover, deletion experiments have shown that the region including both elements is essential for effective transcription of V_κ genes (38). Although the V_H genes do not contain the decanucleotide element, an inverted complementary sequence occurs at the same position (38). Thus, the upstream regions of the J-chain gene and the heavy- and light-chain genes would

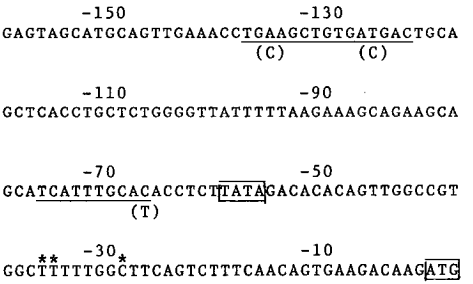

Figure 5 Sequence of the region upstream of the murine J-chain gene. The initiating ATG codon and a TATA box are enclosed in boxes, and the positions of the two transcription initiation sites are shown by asterisks. The conserved elements, the decanucleotide sequence (dc) and the pentadecanucleotide sequence (pd), are underlined; the bases in parenthesis below the underlining indicate differences from the consensus sequences (38).

appear to share elements that are involved in initiating transcription and possibly also in coordinating the expression of the three polypeptides.

The structure of the J-chain gene indicates that it is a simple transcription unit that is activated through changes in DNA conformation. This deduction is supported by the methylation pattern of the J-chain gene (34, 40). Most of the methylation in mammalian DNA occurs at the 5 position of cytosines in the symmetrical dinucleotide, CpG (41). The loss of methyl groups from these sites has been found to correlate with gene activity in a number of eukaryotic systems (42–46). In some cases, CpG sequences become undermethylated throughout the gene (43, 44), whereas in other cases the loss is restricted to a few sites, usually located in the 5′ region of the gene (45, 46). Thus, the extent of hypomethylation can be used as an index of the alterations in chromatin structure required for transcriptional competence.

The methylation status of the J-chain gene has been analyzed by restriction mapping as illustrated in Figure 6. DNA from J chain–negative and J chain–positive cell lines was digested first with the enzyme *Eco*RI and then with enzymes that are inhibited by the presence of methylcytosine within their recognition sites. Southern blots of the DNA from J chain–negative cells showed that all the CpG sites tested were heavily methylated;

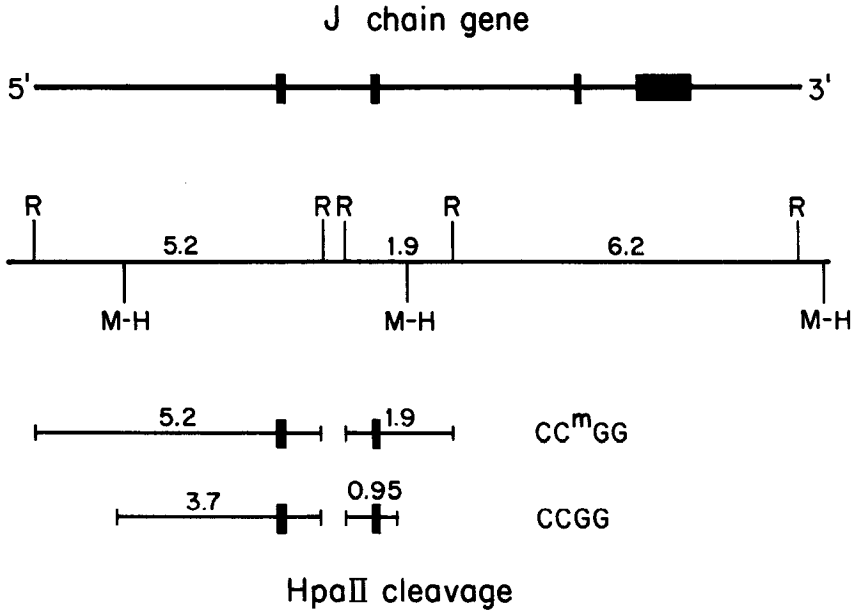

Figure 6 Strategy for assaying methylation of CCGG sequences in the J-chain gene locus. Solid boxes indicate coding sequences. R, *Eco*R1 sites; M-H, *Msp*I/*Hpa*II sites.

only the fragments generated by *Eco*RI cleavage were seen. Conversely, in the DNA from J chain–positive cells the same CpG sites were susceptible to cleavage and gave the patterns expected with complete undermethylation. These findings suggest that transcription of the J-chain gene is associated with chromatin changes that are propagated through the entire gene and the flanking sequences.

How the changes in J chain–gene topology are generated has yet to be determined. From the data available on other eukaryotic gene systems (47–50), it is likely that a B cell–specific regulatory protein(s) provides the signal. Such a protein may bind directly to the 5′ region of the gene to create a site of entry for RNA polymerase II and initiate a cascade of chromatin changes downstream. Alternatively, the regulatory protein may bind to a more remotely located enhancer element and propagate conformational changes to the 5′ polymerase binding site. Although enhancers have been demonstrated in the J-C introns of heavy- and κ light-chain genes (37, 51, 52), a similar element has yet to be identified in the J-chain gene. The noncoding regions examined to date do not show any sequences homologous to the other immunoglobulin enhancers, but because enhancers are known to vary considerably in structure (53), a definitive answer will require assays for enhancer activity.

Synthesis and Regulation of J-chain RNA

By definition, a gene classified as a simple transcription unit codes for a single primary transcript that is processed in an invariant pattern. The products of the J-chain gene conform to this definition. Northern blot analyses (54) have shown that the nuclear RNA from IgM-secreting cells contains a series of J chain–specific sequences, ranging in size from 7.3 kb to 1.3 kb (Table 2). Hybridization with intron-specific probes has identified the

Table 2 Identification of the primary transcript of the J-chain gene and the processed derivatives

Size (kb)	Sequence content				Designation
	Coding[a]	IVS_1[b]	IVS_2	IVS_3	
7.3	+	+	+	+	Primary transcript
5.8	+	−	+	+	1st intermediate
2.7	+	−	−[c]	+	Aberrant splice product
2.3	+	−	−	+	2nd intermediate
1.3	+	−	−	−	Mature message

[a] Coding sequences determined by hybridization with a full-length J-chain cDNA.
[b] IVS = intervening sequences determined by hybridization with appropriate genomic probes.
[c] Negative for 5′ 3 kb of the 3.5-kb second intron sequence.

7.3 kb species as the primary transcript, the 5.8 kb and 2.3 kb species as intermediates produced by the excision of introns in a 5′ to 3′ order, and the 1.3 kb species as the mature message. The 2.7 kb species has been tentatively identified as an aberrantly spliced product generated through the use of an alternative splice site in the 3.5 kb intron. Exactly the same pattern of J-chain transcripts is found in the nuclear RNA of IgG- and IgA-secreting cells. Thus, there are no differences in the synthesis of J-chain RNA that would suggest any differences in J-chain function in these cells.

Differences have been observed, however, between the synthesis of J-chain RNA and μ_s RNA. J-chain precursors are readily detectable in the nuclear RNA from IgM-secreting cells, whereas little or no μ_s precursors can be demonstrated under comparable conditions (54). It is unlikely that this discrepancy in precursor levels is due to differential transcription. Run-on assays of nuclei from IgM-secreting cells have indicated that the J-chain gene has a lower, rather than a higher, rate of transcription initiation than does the μ-chain gene (55). A more likely explanation is that the precursor levels reflect differences in processing rates. Such differences could simply be a function of the particular sequences at the splice junctions of J-chain and μ_s RNA. On the other hand, differential rates of processing could serve as mechanisms for controlling the output of J chain and μ_s messenger RNA.

Analyses of the J-chain sequences in cytoplasmic RNA provide further evidence that J chain is encoded by a single transcription unit. The 1.3 kb RNA identified as the mature message was found to be the single predominant species in all cells examined (56). It accounts for more than 95% of the J-chain RNA in the cytoplasm with traces of the 2.3 kb precursor and a small 0.9 kb fragment making up the remainder. The analysis of J-chain mRNA in IgM-secreting cells also indicate that message turnover plays a significant role in the regulation of J-chain synthesis. The J-chain mRNA content was found to be equivalent to the μ_s RNA content on a molar basis (56), despite the fact that the J-chain gene has a lower rate of transcription initiation than the μ-chain gene and the J-chain RNA transcripts are processed more slowly. To maintain cytoplasmic levels comparable to μ_s mRNA, there must be a compensatory increase in the stability of the J-chain message.

Synthesis and Regulation of J-Chain Protein

The presence of leader sequence information in J-chain mRNA indicates that the J polypeptide is synthesized and processed by the conventional pathway for secreted proteins (57). In this pathway, translation of mRNA is initiated on the free ribosome. Once the amino-terminal leader sequence is synthesized, it mediates the binding of the ribosome to the rough endoplasmic reticulum (RER) and the translocation of the nascent chain

through the ER membrane. On the cisternal side, the leader sequence is enzymatically cleaved and the protein is glycosylated by the transfer of core oligosaccharides to asparagine-X-serine/threonine acceptor sites. The protein is then packaged into vesicles, and processing of the core oligosaccharides and addition of terminal sugars occur as the protein traverses the Golgi apparatus. Direct evidence has been obtained that the J chain follows such a route. Analyses of the mature protein have shown that it lacks a leader sequence (7, 20) and contains an N-linked sugar (58) at the only acceptor site in the sequence, asparagine 49, isoleucine 50, serine 51 (Figure 1). The sugar is of the complex type (59) and varies in terminal sugar content (12). Moreover, analyses of human plasma cells secreting IgM have shown that the J chain is localized in the RER and the Golgi (14).

A different distribution of J chain has been observed, however, in human lymphoid cells representing earlier differentiative stages (14). In null leukemic lines, pre-B lines, and peripheral blood lymphocytes, J chain was detected on free and clustered ribosomes in the cytoplasm, but not in the RER or Golgi. These observations imply that the binding of J-chain ribosomes to the RER is blocked prior to the onset of pentamer IgM synthesis. It is difficult to understand how such a block could be effected. It cannot involve common elements in RER localization, such as the signal recognition unit (60) and the docking protein, (61) because other proteins are transported by the secretory pathway in these cells (14, 62). It is unlikely that some J chain–specific factor is responsible for the block because leader sequences are known to have common recognition structures (57). It is also unlikely that the J-chain leader itself is altered, either by changes at the coding level or by proteolytic digestion of the nascent chain on the ribosome. An explanation of these observations will, therefore, require more detailed analyses of J-chain synthesis in presecreting cells, particularly of the leader and carbohydrate contents of the J-chain product.

As is the case with most eukaryotic proteins, there is no evidence that the synthesis of J chain is regulated at the level of translation. The amounts of J chain synthesized by Ig-secreting cells have been found to correlate directly with the abundance of J-chain messenger RNA (56). There is, however, evidence for posttranslational regulation. Although J chain is synthesized by all mouse myeloma cells (11, 12), it is secreted only as a covalently linked component of the polymers produced by IgM- and IgA-secreting cells (1, 2). Moreover, even in these cells, only a fraction of the intracellular J chain is secreted. Data from pulse chase experiments (12) indicate that the J chain that is not secreted is degraded internally; in each of the various myeloma lines examined, more than 65% of the radioactivity initially associated with J chain could not longer be detected after 2.5 hr of chase. Thus, the degradation of excess J chain appears to serve as a mechanism for

regulating intracellular levels, whether or not the J chain is utilized for polymer assembly.

FUNCTIONS OF THE J CHAIN

A number of different functions have been proposed for the J chain. It has been credited with a primary role in the assembly of Ig polymers (63, 64) and in the differentiative events that lead to pentamer IgM expression (65, 66). It has been attributed a secondary, but critical, role in the secretion of pentamer IgM (10) and in the binding of polymeric IgM and IgA to the transport receptors on epithelial cells (5, 6). In addition, the J chain has been postulated to perform some other, as yet unidentified, function in cells synthesizing monomeric Ig (11, 67). With the increased understanding of J-chain structure and biosynthesis, it becomes possible to take a new look at the evidence for each of these proposed functions.

Requirement of J Chain for Ig Polymerization

A large body of data supports a role for J chain in polymer assembly. Analyses of IgM and IgA structure have shown that one J chain is incorporated per polymer molecule, independent of the size of the polymer (8, 68, 69). The J polypeptide is attached to two polymer heavy chains through disulfide bonds to the penultimate cysteine residues (3, 4). These cysteines are present in the carboxy-termini of secreted μ and α chains (22, 23), but are absent from the carboxy-termini of the membrane forms (71). Thus, the linkage of J chain to Ig heavy chains correlates with the shift to μ_s and α_s expression that is known to accompany polymer Ig synthesis (70, 72–74).

The structural data are reinforced by studies of IgM and IgA polymerization in vitro. Correct assembly was found to require the presence of the J chain (64, 75). Although some reassembly occurs in the absence of the J protein (75–78), the high molecular weight products have been identified as noncovalently bonded aggregates by equilibration with denaturing agents (75). In early studies (64, 75), correct assembly was also found to require a disulfide exchanging system. It is unlikely, however, that an interchange mechanism is responsible for polymer assembly in vivo. Disulfide interchange enzyme, which is synthesized by polymer-secreting cells, has been shown to be active only as a nonspecific protein primer (75, 79). The amounts required for effective polymerization in vitro exceed the levels of enzyme found in polymer-secreting cells. Moreover, other physiological interchange systems, such as mixtures of reduced and oxidized glutathione, are completely ineffective in Ig polymerization (79).

Recently, IgM-secreting cells have been found to synthesize another

enzyme that is associated with the membrane fraction and is highly effective at promoting the IgM polymerization in vitro (80). Less than 1 mole of an enriched enzyme preparation can assemble 30 moles of heavy-light dimers and 3 moles of J chain into pentamer IgM. Analyses of the mechanism of the reaction suggest that polymerizing enzyme is a sulfhydryl oxidase; it resembles Cu^{+2} in catalyzing the formation of IgM intersubunit bonds and, like other known sulfhydryl oxidases, is inhibited by chelating agents. Moreover, analyses of the tissue distribution of the enzyme indicate that it is a specific product of lymphoid cells differentiated to Ig secretion. On the basis of these findings, it would appear that Ig polymers are assembled by an oxidative mechanism that requires not only J chain, but also an enzyme with the specific function of crosslinking monomer and J-chain sulfhydryls.

The evidence from studies of polymer formation in vitro is supported by analyses of pentamer IgM synthesis in rabbit (65, 66), human (81, 82), and mouse (56, 83) lymphocytes. Radioimmunoassays have shown that unstimulated populations from each species contain very small amounts of J chain, 0.05–0.3 ng/10^6 cells, and amounts of monomer IgM consistent with their receptor content. Within 48–72 hours after mitogen stimulation, however, there is a coordinate 20- to 100-fold increase in the levels of intracellular J chain and monomer IgM, and these changes are accompanied by the secretion of pentamer IgM in the culture medium. Similar data have been obtained from analyses of murine cell lines (9, 84). B lymphomas that are representative of unstimulated B lymphocytes were found to lack detectable J chain (Table 3). On the other hand, IgM-secreting myeloma and hybrid lines were found to contain high levels of J chain and monomer IgM in roughly equimolar amounts and actively to secrete a pentamer product (Table 3). The BCL_1 cell line constitutes an interesting exception. The various clones tested expressed large amounts of monomer IgM but very small amounts of J chain. The finding that the cells also secreted very little pentamer indicated that the supply of J chain was the limiting factor in polymer formation (Table 3).

Perhaps the strongest evidence for the essential role of J chain comes from studies of somatic cell hybrids. By fusing a B-cell lymphoma with an IgG-secreting myeloma or any of its nonsecreting derivatives, it is possible to obtain hybrid cells that secrete pentamer IgM (9, 85, 86). Analyses of such hybrids have shown that the polymer IgM is expressed by complementation (9). The hybrid cells retain both the capacity of the lymphoma parent to synthesize monomer IgM and the capacity of the myeloma parent to synthesize J chain, and thus they are able to assemble and secrete a pentamer product.

How absolute is the requirement of J chain for polymer Ig assembly? A polypeptide with the characteristic electrophoretic mobility of the J chain

Table 3 J.-chain synthesis in murine cell lines representing successive stages in B-lymphocyte differentiation

Stage of cell line	Phenotype	Cellular content (molecules/cell × 10^{-5})		Secreted Ig (molecules/ cell/hr × 10^{-5})
		J chain	Ig	
Pre B/B				
ABLS 8	Cytoplasmic μ^+	None[a]	—[b]	None[c]
70Z/3	Cytoplasmic μ^+	None	0.36	None
18-81	Cytoplasmic μ^+	0.18	—	—
B lymphoma				
38C13	Membrane IgM	None	0.53	None
WEHI 231	Membrane IgM	None	3.8	None
X16C 8.5	Membrane IgM	None	0.41	None
BALENLM 17.7	Membrane IgM	None	1.0	None
K46R	Membrane IgM	None	0.58	None
L10A	Membrane IgM	None	0.95	None
IgM secretor				
WEHI 279.1	Immature secretor	2.1	2.2	0.26
BCL_1	Immature secretor	0.05	5.0	0.14
MOPC 104E	Mature secretor	6.5	11	—
M × W 231.1a.2	Mature secretor	11	11	2.5
Other isotype secretors				
Y-8	IgG_3	5.5	—	—
MPC11	IgG_{2b}	4.3	—	—
CBO	IgG_{2a}	10	—	—
W3129	IgA	2.6	—	—
Non-secretors				
Y-8-HC	γ_3	5.2	—	—
CBO-HC	γ_{2a}	7.9	—	—
W3129-HC	α	3.2	—	—
A20	κ	2.1	—	—
M12	κ	3.1	—	—
P3/NS1/1-Ag4-1[d]	κ	5.3	—	—
No Ig expression				
SP2/0-Ag14[d]	None	6.3	—	—
S194/5.XX0.Bu.1[d]	None	3.5	—	—

[a] None represents less than 300 molecules/cell, the limit of detection of the J-chain radioimmunoassay.
[b] Dash = not determined.
[c] None represents a secretion rate of less than 1000 pentamer molecules/hr.
[d] Plasmacytomas commonly used as fusion partners.

has been detected in the reduced and alkylated polymer immunoglobulins of many mammalian species, in several species of birds, and in some amphibians (reviewed in 15). It has also been detected in the immunoglobulins from bony fishes and the more primitive cartilagenous fishes where pentamer IgM is the only functional antibody synthesized. Two exceptions have been described. The normal macroglobulin from the gar (87) has been reported to lack detectable J chain, and a number of monoclonal IgM and IgA proteins (6, 88–92) isolated from the serum of human patients have been found to lack J chain or to contain variable amounts. These findings do not necessarily show that the requirement for J chain can be bypassed. Because the J polypeptide is particularly susceptible to proteolysis (17), degradation could account for the observed losses of J chain. Thus, there is no convincing evidence that J chain is not essential for Ig polymerization.

Proof of a mandatory role, however, will require resolution of the mechanism of polymerization. Analyses of the reductive cleavage products of IgM and IgA have indicated that the J-heavy bonds are more stable than the heavy-heavy intersubunit bonds (8, 69, 93). On the basis of these data, it has been postulated that J chain initiates polymer assembly by forming a disulfide bridge between two monomer subunits (8). In the case of IgM, the resulting J chain–containing dimer would serve as a nucleus for the addition of three other monomers. In the case of IgA, the J chain–containing dimer would be secreted directly from the cell. This hypothesis is supported by the observed stoichiometry of one J chain per polymer and by the available data on J-chain linkage (8, 69, 94). However, the alternative, but less likely, possibility that J chain links two heavy chains within a single monomeric subunit has not been rigorously excluded (95).

The mechanism of polymer bond formation is also open to question. In the past the disulfide bonds were thought to be generated through a series of exchange reactions (64, 75), but the recent studies of a lymphocyte polymerizing enzyme suggest the S-S bonds may be formed by direct oxidation (80). Hopefully, this question can be resolved when the enzyme is purified and its activity is examined in a defined in vitro system. It will be of considerable interest to determine whether the enzyme specifically binds the J chain. Such a finding would provide direct evidence both for the role of the enzyme and for the requirement of J chain in polymer Ig assembly.

Role of J Chain in B-Cell Differentiation

The pattern of J-chain expression in mitogen-stimulated lymphocytes suggested that de novo synthesis of the J chain might be a critical step in the differentiation of a B lymphocyte to an IgM-secreting cell (65, 66). In the mouse system (10, 56), this hypothesis has been substantiated by comparing

the expression of J-chain RNA in cell lines representing successive stages in the differentiative pathway (Table 3). No J chain–specific sequences could be demonstrated in Northern blots of lymphoma RNA regardless of the maturation stage of the lymphoma line, whereas large amounts of J-chain RNA were detected in Ig-secreting lines. From the conditions used for hybridization, it was calculated that the limit of detection of the analyses was one J-chain transcript per ten cells. A second line of evidence (56) has been obtained by following the expression of J-chain RNA in normal lymphocytes stimulated with bacterial lipopolysaccharide (LPS). The initial unstimulated population was found to contain a low level of J chain–specific RNA which could be accounted for by the few secreting cells that remained after fractionation. At 48 hr after LPS stimulation, the residual J-chain transcripts were no longer evident, and at 72 hr newly synthesized J-chain RNA appeared and rapidly accumulated in the cytoplasm. The sum of these data indicates that transcription of the murine J-chain gene is initiated in the stimulated B cell.

In the human system, however, there is evidence that J-chain synthesis is initiated at an earlier stage in differentiative pathway (14). As was discussed in a previous section, J-chain protein has been detected by immunoelectron microscopy in cells from patients with acute lymphocytic leukemia of the null and pre-B types, as well as in cells from two cloned human pre-B lines. Quantitation of the J-chain content showed that the levels in the leukemic cell populations exceeded those found in normal unstimulated lymphocytes, whereas the levels in the pre-B lines were similar or lower.

There are several possible explanations for the discrepancy between the mouse and human data. The earlier expression of J chain in human B-cells could represent an evolutionary development. The human hematopoietic system may have acquired a mechanism for inducing constitutive synthesis of J chain at the time when heavy and light chains are first expressed. The advantage of such a mechanism to the human organism remains to be determined. Alternatively, the synthesis of J chain in null and pre-B lines could be a consequence of malignant transformation. Of three murine pre-B/B-cell lines that have been examined for J-chain synthesis, two—the 70Z/3 line and the Abelson-transformed ABLS 8 line—were found to be negative, and the other, the Abelson virus transformed 18-81 line, expressed very small amounts of J chain mRNA and protein (Table 3). Finally, J-chain positive lines classified as null or pre-B could represent cells that were transformed at the IgM-secreting stage and, as a result of the transformation process, retained the capacity to synthesize J chain but lost the capacity to express heavy and light chains. This possibility is consistent with the observations (62, 96) that many null leukemic cells have rearranged heavy- and light-chain genes (often in an aberrant form) and synthesize

essentially no Ig products. To determine which, if any, of these alternative interpretations is correct will require more extensive characterization of both murine and human precursor B lines.

Role of J Chain in Polymer Ig Secretion

There is considerable evidence that the secretion of IgM is dependent on polymerization and thus indirectly on the presence of the J chain. Analyses of murine lymphoid lines have shown that all IgM-expressing cells synthesize both secreted and membrane forms of μ mRNA (70, 72, 73) and the corresponding μ_s and μ_m polypeptides (72). The secreted form constitutes 40–50% of the intracellular μ chains in B lymphomas representative of immature and mature B cells and 70–90% of the μ heavy chains in IgM-secreting plasmacytomas (56, 72). Yet little or no IgM of the secreted type is released from the lymphoma cells (9, 97–99); IgM is secreted only from the plasmacytoma cells when the μ_s translation product is assembled with J and light chains into pentamer molecules (84). A similar pattern is seen in normal B cells stimulated with mitogens or T-cell factors (56, 66, 72, 83, 100). The secreted form of μ_s mRNA is synthesized during the early stages of the response, but IgM is not secreted until a later stage when J-chain synthesis is initiated and amplified (56, 83).

The selective secretion of pentameric IgM indicates that the B cell distinguishes between μ_s polymers and μ_s monomers and targets the polymers to a secreted pathway and the monomers to an intracellular route that leads to their degradation. How the distinction is made remains to be resolved. The recognition could be based on structural differences between monomeric and aggregated Fc domains to which the J chain might contribute indirectly; alternatively it could be based on the presence or absence of the J chain itself. Studies of hybridomas producing mutant μ chains (101, 102) suggest that Fc structure is a critical signal in monomer recognition; μ chains lacking the C2 domain or the C2 and C3 domains were found to be secreted as H-L hemimers whereas μ chains lacking the C4 domain were secreted in very small amounts as H_2-L_2 monomers. Thus, deletion of any Fc domain appears to destroy the monomer recognition signal and allows the truncated μ-L complexes to be secreted, probably by bulk phase movement from the Golgi (103). What effect deletion of the C2 and/or C3 domains has on the polymer recognition signal could not be determined. Polymer formation is blocked in these mutants because the light chains form disulfide bonds with the heavy-chain cysteines normally involved in intersubunit linkage.

In contrast to the secretion of IgM, the secretion of IgA is clearly independent of polymerization. Normal lymphoid tissues have been shown to secrete both monomer and dimer IgA, the relative amounts depending

on the particular location of the tissues (104–106). Moreover, cloned lines secrete monomeric and dimeric IgA, and even individual cells may produce both forms (71, 74, 107). This behavior suggests that either the IgA-synthesizing cell secretes monomeric and polymeric products by separate pathways or it employs a single pathway that does not discriminate between the two species.

Role of J Chains in Polymer Effector Functions

Monomer IgM and monomer IgA acquire specialized effector functions as a result of their respective polymerization. Of these functions, only one, the transcellular transport of polymeric IgM and IgA into the external secretions, has been found to depend on the presence of the J chain (5, 105). The transport is mediated by receptors that are located on the internal surface of most glandular epithelium and specifically bind the polymeric Ig products of the local lymphoid tissue (108, 109). The receptor-ligand complexes are endocytosed, transported across the cell in vesicles, and then released into the body cavities (110–112). During this process, the ligand-binding domain of the receptor, the secretory component (SC) (113–117), is proteolytically cleaved (118, 119) and discharged in association with the polymeric Ig. Studies of the interaction of polymer Ig with free SC (6) or with receptors on epithelical cell lines (120) have shown that the extent of binding correlates with the J-chain content of the ligand. Polymer IgM and IgA containing the normal complement of J chain reacted quantitatively, whereas polymers depleted of J chain bound poorly, and monomer species exhibited no significant binding. As is the case for the polymer recognition site for pentamer IgM secretion, the polymer recognition site for transcellular transport has not been identified; it may involve the J chain itself or Fc structures that are generated as a result of J-chain association. The latter is the more likely possibility, in view of the relative inaccessibility of the J chain in the polymer structures (121) and of the inability of free J chain to inhibit the polymer binding to SC (122).

Role of J Chain in Monomer Ig-Secreting Cells

Perhaps the least understood feature of J-chain expression is its synthesis by monomer Ig-secreting cells. Analyses of murine myeloma lines have indicated that all monomer Ig–secreting cells and their derivatives express the J chain (11, 12, 84, 123). The derivatives include mutant cells that synthesize only heavy chain, only light chain, fragments of light chain, and even fusion partners that synthesize no detectable immunoglobulin (Table 3). The levels of intracellular J chain in these lines range from 10^4 to 10^6 molecules per cell, values comparable to those obtained for cells actively secreting polymeric IgM or IgA. Normal plasma cells, however, exhibit a

different pattern of J-chain expression (81, 124). Fluorescent staining (125, 126) has shown that 40–70% of the IgG-secreting cells in human glandular tissue or in active germinal centers are J chain–positive, whereas only a small percentage of the IgG-secreting cells in extrafollicular sites or in chronic foci of inflammation contain detectable J chain. Moreover, analyses of human peripheral-blood cells producing various IgG subclasses (127) suggest that J-chain expression is related to the maturation of monomer Ig-secreting cells. The frequency of J chain–positive cells was found to decrease as a function of the 5′ to 3′ order of the γ subclass gene expressed.

These findings raise two questions. First, how can the differences in J-chain content between normal and transformed monomer Ig-secreting cells be explained? Although immunofluorescence is a less sensitive method of detection than those used for the analyses of transformed lines, it is unlikely that the lower sensitivity could account for the magnitude of the observed differences and the skewed tissue distribution of J chain–positive IgG-secreting cells. It seems more likely that the higher levels of J chain seen in monomer Ig-secreting myeloma cells are a function of their neoplastic state. The transformation process could cause an increase in J-chain synthesis, or the induction of myeloma tumors in the peritoneal cavity could selectively transform those monomer Ig-secreting cells in the intestinal mucosa that express relatively large amounts of J chain.

The second and more important question is: How can the synthesis of J chain in monomer Ig-secreting cells be explained? There is no evidence that J chain influences the assembly of monomeric immunoglobulins (12, 128, 129) or plays a role in their secretion (67). One possible explanation is that cells of the B lineage have no mechanism for shutting off J-chain synthesis once it is initiated during the pentamer IgM response. The lack of such a mechanism could have several advantages. It would allow the plasma cell to switch to the synthesis of polymeric IgA without the necessity of reactivating J-chain expression (11). It would also allow the J chain itself or some product of its synthesis to promote the switch to IgA expression. Studies of fetal liver and splenic B-cell clones (130) have shown that a disproportionally large fraction express IgA, much larger than would be expected from successive 5′ to 3′ switches at the heavy-chain locus. Moreover, recombinational events in immunoglobulin genes are known to be regulated by product feedback (131); for example, analyses of pre-B cells indicate that the production of a functional μ heavy chain is necessary for the initiation of V-J recombination at a light-chain locus (132, 133). In the absence of a specific repressor mechanism, the reduced levels of J chain seen in some monomer Ig-secreting cells would have to be attributed to nonspecific changes in the rates of J-chain synthesis or degradation.

A second possible explanation of the data is that B cells have developed a

mechanism for repressing J-chain synthesis that serves as a signal for the generation of IgG memory cells. The IgG-secreting cells with high J-chain contents would then represent a population of plasma cells that switched directly from the synthesis of pentamer IgM to the synthesis of monomer IgG. On the other hand, the IgG-secreting cells with little or no cytoplasmic J chain would be derived from memory cells that were recalled to active secretion by a secondary encounter with antigen. This interpretation is consistent with the low frequency of J chain–positive IgG-secreting cells in areas of chronic inflammation and with the poor memory response exhibited by the secretory IgA system.

The available data do not allow any distinction to be made among these various possibilities. Moreover, studies of J-chain function at the later stages of B-cell differentiation are hampered by a lack of information on the genetics of the J chain and by a lack of appropriate cell lines, i.e. J chain–negative fusion partners, mutants expressing altered forms of J chain, well-defined representatives of memory cells. Hopefully, it will be possible to circumvent these difficulties by the use of gene transfection methods and thus resolve the issue of whether intracellular J chain in IgG-secreting cells represents "a relic from previous commitment to IgM synthesis" (11) or serves positive regulatory function(s) in the differentiation of IgG-secreting cells.

SUMMARY

During the last decade the immunoglobulin J chain has indeed come of age. The amino-acid sequence has been determined for the mouse and human polypeptides, and the data obtained have established the uniqueness of the primary structure and its high degree of conservation in vertebrates. The biosynthesis of J chain has been defined: The information is encoded in a simple transcription unit that is induced by changes in chromatin structure to express a single primary transcript and a single mature message; translation of the message yields a propolypeptide which is processed and transported through the cell by the conventional pathway for secreted proteins. As a result of these advances, many of the functions of the J chain have been clarified. Analyses of the expression of J chain mRNA and protein in mouse lymphocytes have shown that the initiation and amplification of J-chain synthesis are critical steps in the pentamer IgM response because the J polypeptide is required for the assembly of the IgM antibody. Analyses of the behavior of the polymeric Igs have established that the J-chain component contributes, certainly indirectly and perhaps also directly, to the secretion of pentamer IgM and the transcellular transport of both IgM and IgA.

However, knowledge of the J chain has yet to achieve full maturity. How

the J chain participates in the polymerization of IgM and IgA needs to be reexamined in view of the recent findings that assembly may involve an oxidative mechanism catalyzed by a lymphocyte-specific enzyme. The observation that J chain may be constitutively expressed in human B cells and the possibility that J chain performs additional regulatory functions in monomer-Ig secreting cells should be pursued. Determination of the J-chain secondary structure and its arrangement in the polymer Fc domains is critical to understanding the effector functions of polymer Ig. Lastly, the finding that late-acting factors, such as interleukin-2, induce an amplification in J-chain synthesis opens the way to using the expression of J chain as a model system for dissecting the mechanism of gene regulation. These are the challenges for the next decade.

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CONTENTS

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EFFECTOR AND REGULATORY MECHANISMS IN IMMUNITY TO SCHISTOSOMES: A Heuristic View

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INTRODUCTION

Parasitic diseases represent a significant burden for health and for socioeconomical development in many tropical countries. The immunoprophylaxy of these infections would be a major advance, and this certainly justifies the important investments made at national and international levels in the search for a vaccine against the major human parasites. It should be stressed, however, that despite remarkable and sometimes spectacular progress, this goal still remains far out of reach. Parasites are, in general, of very ancient lineage; schistosomes for instance evolved from blood flukes during the Permian era, which means that they have had something on the order of 250 million years to perfect survival tactics appropriate to their coexistence with their vertebrate host immune system. For evolutionary reasons, in comparison with other infectious agents, they represent by their structure and the complexity of their life cycle a highly elaborate model of host-parasite interplay. Not surprisingly therefore, when immunologists started to study the immune response to parasites, they discovered that the adaptation of the parasite to its host was the result of a complex, refined, and dynamic balance between host effector mechanisms and parasite escape strategy. At the same time, they were struck by the fact that some entirely novel mechanisms were involved in antiparasite immunity, almost allowing researchers to rewrite some chapters of immunology textbooks.

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The purpose of this review is to give an account of the immunology we have learned from parasites. The general message is that the understanding of these mechanisms appears indispensable for possible development of antiparasite vaccines and, more generally, provides new and fascinating insights into many areas of human pathology.

Among the parasites used as models, schistosomes probably have been employed in the broadest experimental approach. They have illustrated the existence of novel effector and regulatory mechanisms among the immunological components of the specific response to metazoan parasites.

An infection that affects 300 million people in the world, schistosomiasis is characterized by the presence of adult worms in the portal and mesenteric veins of humans and of various other mammalian species, as part of a complex migratory cycle initiated by cutaneous penetration of aquatic infective cercariae. These transform into schistosomula under the skin of appropriate hosts. It is generally agreed that pathological reactions to schistosome infection are related to the deposition of numerous parasite eggs in host tissues (106).

STUDY MODELS

Although clinical studies have made possible the investigation of several immune mechanisms operating in man (17, 78), most of the information is derived from the use of various experimental models, among which the mouse and the rat have been the most widely used. Rat infection by schistosomes deserves a particular mention. In this rodent, experimental infection by cercariae results in the establishment of a worm population that is almost entirely rejected three to four weeks after the initial exposure; this rejection itself is followed by a strong and prolonged immunity to reinfection. The comparison of immune responses to schistosomes in the mouse and in the rat reveals striking differences between these two models.

Whereas nonspecific factors of resistance (natural killer cell activity, activated macrophages resulting from inflammation, tissue damage and hemodynamic changes elicited by worms or their eggs) can be easily demonstrated in the mouse, significant evidence for a specific protective immune response is seldom obtained in this model. Conversely, the rat seems to provide the opposite example since, apart from complement activation, very little evidence if any has been obtained for nonspecific mechanisms, while B-cell depletion (12) or passive transfer experiments with polyclonal (26) or monoclonal (24, 53) antibodies clearly indicated the prominent role of humoral immunity. Interestingly, the cytotoxicity systems we will describe in the rat model have not been clearly shown in mouse schistosomiasis but have been precisely demonstrated in human

infection. We therefore concentrate this review on those mechanisms that have been identified in both rat and man, and have allowed through transfer experiments the assessment of their in vivo significance.

COMPONENTS OF THE IMMUNE RESPONSE TO SCHISTOSOMES

Much experimental evidence supported by epidemiological observations in humans points to the invasive stage of the parasite, i.e. schistosomulum, as the main target of immunity. The adult population seems relatively unaffected by immune effector mechanisms, which led Smithers & Terry (92, 93) to the concept, now clearly established, of concomitant immunity.

A brief analysis of the various components of the immune response reveals some essential features:

1. Schistosome infection elicits a very intense humoral response, among which the massive production of anaphylactic antibodies is striking (42, 81, 82).
2. As soon as they acquire a functional gut (8–10 days after penetration), juvenile worms and adults release large amounts of their metabolic products into circulation in their hosts, where circulating antigens and immune complexes can then be detected (27, 61, 84, 96).
3. Much evidence has accumulated concerning the thymodependency of some essential components of the immune response (IgE production, hypereosinophilia, delayed-type hypersensitivity) (30, 77).
4. It has been demonstrated, however, that cytotoxic T cells are not involved in killing mechanisms (18, 90).
5. On the other hand, all the experimental results obtained by serum transfer (26, 90) or in anti-μ-treated animals (12) point to the antibody response as an essential factor of immunity.
6. In vitro experiments show that antibody-dependent, cell-mediated cytotoxicity (ADCC) appears as a primary mechanism of defense against schistosomes (21).
7. One of the main characteristics of these ADCC systems is that they do not involve conventional lymphoid cells but rather phagocytic cell populations, among which the participation of eosinophils and macrophages is particularly evident.
8. Finally, only the young schistosomula appear susceptible to the various killing mechanisms, and the parasite appears to lose its susceptibility to immune attack within a period of two to three days (37, 44).

Before going into a detailed analysis of effector mechanisms and their regulation, I think it important to emphasize that immunity to reinfection

in schistosomiasis, as in many parasitic diseases, appears to be a multifactorial process involving several antibody isotypes, complement factors, and various cell populations not necessarily acting all together at a given time. One should also remember that immunological studies of a parasitic model have to be placed and interpreted in the context of the natural history of the parasite infection, which involves a dynamic process of maturation and development of the worm. Obviously, the complexity of this network makes a precise in vivo analysis of the mechanisms involved extremely difficult. During recent years considerable investment has been made in in vitro studies that may not reflect the complexity of the in vivo situation in all cases, but that have had the advantage of elucidating the potential mechanisms of killing. These in vitro studies have also indicated that the relative in vivo inefficiency of the killing processes may be related to immunoregulatory or escape mechanisms of the parasite; these are also considered in this review.

EFFECTOR MECHANISMS OF NONSPECIFIC IMMUNITY

Complement activation through the alternative pathway at the level of the schistosomulum membrane was shown to be responsible for significant killing (85) and appears as one of the possible mechanisms of nonspecific immunity. This cytocidal activity of complement can even be amplified when cells bearing C_{3b} receptors, e.g. normal eosinophils, are activated in the presence of complement but in the absence of specific antibody (2, 69, 80). The potential role of complement is supported by the fact that acute complement depletion by cobra-venom factor significantly reduces immunity to reinfection in rats (87).

The presence on the membrane of schistosomula of C_{1q} (75, 86) and C_{3b} receptors (76, 83) is also worth mentioning since these structures can act as inhibitors of the cascade of complement activation (50, 57) and therefore represent a potential evasive mechanism for the parasite. Natural killer (NK) cells and increased NK-cell activity have also been reported in mouse schistosomiasis (1, 3).

More extensive information has been gained recently concerning the killing capacity of lymphokine-activated macrophages (14, 59, 64, 73). Although it is clear in the mouse, the real significance of this mechanism appears unsettled in man, since monocyte killing activity by itself is decreased in human schistosomiasis (74). Finally, the possibility of inducing a strong resistance to infection or reinfection by various nonspecific immunostimulants like BCG, cord factor, or muramyl dipeptide, or by the

elicitation of inflammatory reactions by chemical or bacterial agents must be kept in mind (13, 66).

SPECIFIC EFFECTOR MECHANISMS

When it was discovered twelve years ago that schistosomula could be killed in vitro by immune serum from monkeys (37), nobody could predict that this was the beginning of a great era of research concerning potential effector mechanisms against schistosomes. Indeed, in the past ten years, not only were many known cytotoxicity systems shown to be effective, but several novel mechanisms were also described. From this accumulation of information, two important features emerge: (*a*) the failure of T cells to kill, and (*b*) the evidence for antibody and cell collaboration in cytotoxicity against schistosomula.

Failure of T Cells to Kill

The possibility that a T cell–mediated killing process could exist has been carefully investigated. The occurrence of cytotoxic T lymphocytes (CTL) was all the more plausible since MHC (major histocompatibility complex) products of the *K/D* and *I* loci were shown to be acquired by schistosomula (51, 89) and thus, in principle, all necessary conditions for CTL activity were fulfilled. Even though alloreactive or hapten-specific cytotoxic T cells of the Lyt 1^-,23^+ phenotype were shown to be capable of adhering specifically to schistosomula, these remained undamaged (18, 104). A more recent report claiming killing by T cells in human schistosomiasis (49) has not been confirmed. One can only speculate at this stage on the reasons for the failure of potential CTL to induce killing, but it very likely indicates that although recognition and adhesion can take place, some escape mechanisms prevent or affect the efficiency of the lytic process at the CTL-parasite interface.

ADCC as Primary Effector Mechanisms of Defense

Contrasting with the inefficiency of T cell–mediated killing, antischistosome antibodies can damage schistosomula in vitro. The initial observation made with Rhesus monkey immune serum led to the identification of an IgG antibody lethal for schistosomula in the presence of complement (37). This was rapidly followed by several pieces of evidence indicating that complement-dependent antibodies could achieve a highly significant killing of schistosomula in various animal species including man, rabbit, rat, and mouse (22, 29, 71). Yet, the exact biological significance of the so-called lethal antibody is so far unsettled.

Following this early demonstration, most of the numerous studies

indicated that antibody-dependent, cell-mediated cytotoxicity (ADCC) appeared as a potent mechanism of killing schistosomes. One of the most fortunate factors for immunoparasitologists in the study of the effector mechanisms of immunity to schistosomes certainly arose from the fact that conventional lymphoid cells were not involved in cytotoxicity reactions. This contributed significantly to the knowledge and understanding of ADCC mechanisms in general by showing that contrary to the immunological situation in tumors or allografts, inflammatory cells and platelets were the essential cellular factor (Table 1).

A second prominent feature was the demonstration that besides IgG antibodies initially shown to interact with eosinophils (15–17) or neutrophils (39, 58). IgE antibodies represented a major humoral factor in cell activation leading to schistosomula killing. This was first assessed by the observation that normal rat macrophages or normal human monocytes could be triggered into highly efficient killer cells in the presence of specific IgE antibodies (23, 27, 61).

A clearcut demonstration of the essential participation of IgE antibody in macrophage-mediated killing of schistosomula was obtained by selective IgE depletion from immune serum or by the use of a monoclonal antischistosome IgE antibody (Figure 1) (24, 27, 61).

Similar observations were made in eosinophil-mediated cytotoxicity. Indeed, after six weeks of infection, during which IgG antibody could almost exclusively be incriminated in the rat, IgE antibody played a major role in triggering the cytotoxic function of eosinophils (28). It is noteworthy that even during the first period of rat infection when IgG antibody is involved, the subclass was identified as IgG_{2a} (32)—the other anaphylactic isotype in this species. Again, immunoabsorption experiments with various anti-rat immunoglobulins and the use of monoclonal antischistosome antibodies of the IgG_{2a} or IgE isotypes (24, 53) provided clear evidence of the general role of anaphylactic antibodies in eosinophil-mediated killing.

Table 1 Main characteristics of ADCC against schistosomes

Effector cells	Antibody isotype		Accessory stimulus	Correlation with immunity in rats (weeks postinfection)
	Humans	Rats		
Eosinophils	IgG	IgG_{2a}	Mast cell	4–6
Eosinophils	IgE	IgE	Mast cell	6–13
Macrophages	IgE	IgE	No	6–13
Platelets	IgE	IgE	No	5–7
Neutrophils	?	IgG	Complement	Unknown

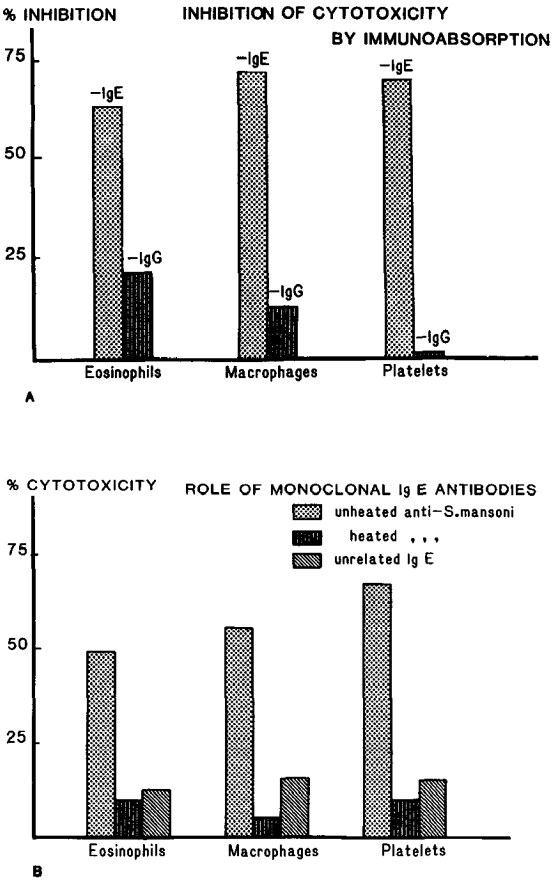

Figure 1 Role of IgE antibody in cell-mediated cytotoxicity to schistosomes. (*a*) Selective immunoabsorption by various antirat immunoglobulins of immune rat serum (6 weeks postinfection) prior to incubation with the three effector cells studied. (*b*) Eosinophil-, macrophage- and platelet-mediated cytotoxicity induced by a rat IgE monoclonal antibody to *S. mansoni* [data from (24)].

These observations of IgE-dependent killing by rat eosinophils were confirmed recently in human schistosomiasis (35).

In addition, a most exciting recent finding has been that rat platelets in the presence of immune rat serum could kill schistosomula in vitro. Various experimental procedures including immunoabsorption of IgE, inhibition with aggregated myeloma E protein, and the use of monoclonal antischistosome IgE antibody indicated the IgE dependency of this novel ADCC system (Figure 1). Interestingly, normal human platelets could be turned

into cytotoxic effectors by the addition of infected human serum, a process that could be almost abolished after selective depletion of IgE (60).

In summary therefore, it appears that three cell populations—macrophages, eosinophils, and platelets—are essentially involved through their interaction with IgE antibody, in specific killing of schistosome larvae. The existence of such IgE-dependent cellular cytotoxicity mechanisms raised among many others the problem of the existence of specific IgE receptors and of their role in triggering effector cells.

Fc_ε RECEPTORS AND IgE-DEPENDENT CELL ACTIVATION

It is not the purpose of this review to analyze the work that led to the characterization of Fc_ε receptors, so far unknown, on macrophages, eosinophils, and platelets, but it seems possible to summarize some of their essential properties with respect to their role in cell activation.

At variance with the classical receptor of mast cells and basophils, characterized by high-affinity monovalent binding, all the information available concerning macrophages, eosinophils, and platelets indicates common IgE receptor characteristics distinct from the above mentioned: (*a*) low-affinity binding (average equilibrium association constant in the range of 10^7 M^{-1}); (*b*) preferential interaction with aggregated IgE molecules; (*c*) average of 5×10^4 receptors per cell; and (*d*) this group of low-affinity receptors has a common antigenic specificity shared with lymphocytes, but distinct from mast cell or basophil receptors (25, 70).

The direct participation of Fc_ε receptors in the induction of the cytotoxic activity of macrophages, eosinophils, and platelets was clearly demonstrated in these three ADCC systems, by a dramatic decrease in parasite killing after the addition of an anti-Fc_ε receptor antibody (24, 35) (Figure 2).

The involvement of the complexed form of IgE antibody was shown by several observations. As already stated, circulating IgE immune complexes were detected in the serum from infected humans or experimental animals (84, 96). The involvement of the complexed form of IgE antibodies was indicated by the triggering of the ADCC reactions by the ultracentrifugation pellets from immune sera (21, 27), and further substantiated by inhibition experiments with various molecular forms of purified E myeloma protein, showing that only preincubation of each of the cells with aggregated IgE could block the killing process. The exclusive role of IgE antibody in triggering the cellular cytotoxicity was supported by the absence of inhibition by aggregated IgG (27, 28, 60, 61) (Figure 3).

It is generally accepted that in ADCC reactions, cytocidal factors are released by activated effector cells. This appears to be the case of ADCC

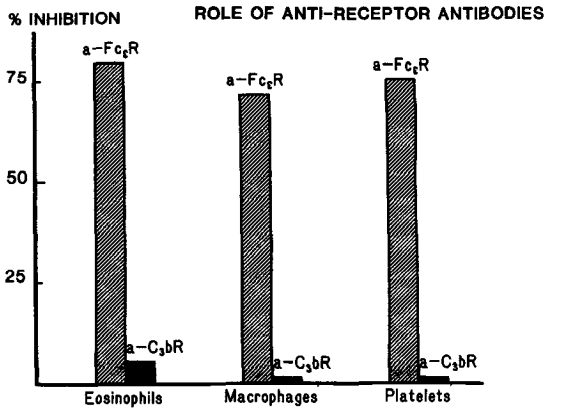

Figure 2 Inhibition of ADCC to schistosomes by an antireceptor antibody (70) that blocks binding of IgE to eosinophils, macrophages, and platelets [data from (24, 35)].

against schistosomes. Although no conclusive evidence of the predominant importance of a given factor has been developed, there is a great deal of experimental evidence for the release—by macrophages for instance—upon IgE-dependent activation, of lysosomal enzymes, oxygen metabolites, or cytocidal proteins (41, 62). Again, cell activation requires a minimal degree of aggregation of IgE molecules, at least as dimers in the case of macrophages; the binding of dimeric IgE is followed by a rapid increase in cyclic GMP in a calcium-dependent process (43).

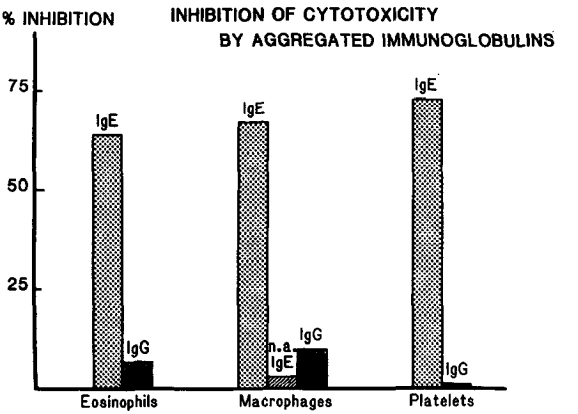

Figure 3 Inhibition of eosinophil-, macrophage-, and platelet-mediated cytotoxicity to schistosomes by adding to 6-week-infected rat serum either aggregated rat myeloma IgE or IgG (n.a. = nonaggregated Ig).

The evidence for IgE-dependent cell activation in these ADCC systems does not exclude the participation of other activating signals. In eosinophils in rat schistosomiasis for example, in addition to the signal provided by the binding of the Fc portion of IgG_{2a} or IgE antibody to the surface receptor, the presence of mast cells or of their degranulation products was required to achieve a significant degree of cytotoxicity by eosinophils (32, 34). Among potential factors released by mast cells upon their activation by anaphylactic antibodies, ECF-A tetrapeptides could reproduce in vitro the effect of the accessory mast cell population (31). These findings were confirmed recently with human eosinophils (35). It was also shown that parasite-derived enzymes can directly increase the killing capacity of normal eosinophils (4, 5).

In a given cell type the expression of Fc_ε receptors, as estimated by IgE-rosette assays, appears as a dynamic process during infection, reaching its maximum when IgE-dependent, cell-mediated cytotoxicity is observed (28); it is also related to the heterogeneity of the cell populations. In the case of eosinophils, there is evidence that after purification on metrizamide gradients, low-density eosinophils express the highest number of Fc_ε receptors together with the highest killing capacity (35).

In summary therefore, and as far as the rat model is concerned, three cell populations and two anaphylactic antibody isotypes, IgG_{2a} and IgE, appear as the essential components of in vitro effector mechanisms. Although these components are also found in the study of ADCC reactions in man, no information is at present available concerning the IgG subclass involved. Yet experiments performed in the rat model confirm the particular biological importance of anaphylactic antibody isotypes in immunity to schistosomes.

IN VIVO RELEVANCE OF THE EFFECTOR SYSTEMS

One must first note that there is a close chronological relationship between the development of protective immunity to reinfection by schistosomes in rats and the evolution of anaphylactic antibodies and anaphylactic immune complexes, and the dynamics of in vitro cytotoxicity assays (21). On the other hand, selective depletion of either IgG_{2a} or IgE from immune rat serum leads to a significant decrease in passively transferred immunity (26). More conclusive evidence was obtained by transfer experiments using monoclonal antischistosome antibodies. Both IgG_{2a} and IgE monoclonals are indeed able to confer to recipient animals significant immunity to a challenge infection (24, 53).

These findings tend to support the biological function demonstrated in

vitro of these two anaphylactic antibody isotypes, but they do not allow the attribution of any particular function to a given cell population. Recent experiments, however, provided suggestive evidence for the in vivo participation of eosinophils, macrophages, and platelets. In the case of eosinophils, a significant degree of protection resulted from purified eosinophils collected at the early stage of immunity and passively transferred to naive rats at the site of the infection (33). These findings must be related to previous observations that showed the existence of cytophilic antibody on the surface of eosinophils from infected animals and the direct in vitro killing activity of these cells (36); they are consistent with both the identification of eosinophils around damaged worms (56, 105) and the reported ability of anti-eosinophil serum to decrease reinfection immunity in the mouse (67). Confirming the results with in vitro cytotoxicity systems, both eosinophils and macrophages from immune rats could later on transfer resistance passively to a challenge infection (33). Interestingly, a highly significant degree of protection can be transferred to normal syngeneic rats with platelets from immune donors collected at the optimum period of immunity (60).

As fragmentary as they are, these in vivo experiments clearly confirm, at least in rats, the biological function of the antibodies and the cells identified by in vitro assays in man and rat. Within the limits of the present discussion, in vitro systems appear thus to be good correlates of in vivo mechanisms.

One of the major drawbacks of in vitro assays, however, remains that they give a valid but isolated and simplified picture of a given component of the immune mechanisms. It is now clear from the in vivo analysis of effector systems against schistosomes that they are the result of multifactorial processes involving several cytotoxicity mechanisms either successively or synergistically (24). One of the most beneficial consequences of these observations is the recent characterization of functional antigens at the surface of schistosomula, which appear as potentially protective antigens.

TARGET ANTIGENS

Characterization of Schistosomulum Target Antigens

Several works demonstrate the complex composition in protein and glycoprotein of membranes isolated from larval or adult stages of *Schistosoma mansoni*. Immunoprecipitation experiments also provide evidence of the existence of membrane antigens common to different stages of the parasite. Using monoclonal antibodies, researchers showed that spine glycoproteins of cercariae and adult worms expressed a common epitope but differed in molecular weight: 120 kDa in cercariae and 170 kDa in male and female worms (72).

The characterization of antigens exposed at the parasite surface was mainly investigated by surface-labelling with iodine. The first results concerning the surface-labelling of adult schistosomes were reported by Hayunga et al (55), who showed that the Bolton-Hunter technique was the most suitable for the labelling of adult worm surface components. Snary et al also observed the relative inefficiency of the lactoperoxydase technique on the adult stages of schistosomes (94). The ^{125}I-iodosulfanilic acid (101) or lactoperoxydase technique appeared very useful for the extrinsic labelling of the larval surface, allowing the identification of major schistosomulum surface antigens of 30–40 kDa and 15–25 kDa, recognized by serum obtained from chronically infected animals or humans and from animals immunized with irradiation-attenuated cercariae (46, 88, 91, 97, 99).

The search for characterization of schistosomulum functional antigens was carried out with monoclonal antibodies that could protect animals against a challenge infection. A 130-kDa component was precipiated from KCl-extracts of ceracriae by mouse protective monoclonal antibodies (107), which suggests that this antigen could be involved in immunity to schistosomes. The particular use of the rat IgG_{2a} monoclonal antibody to induce a high degree of protection by passive transfer has allowed the characterization of a 38-kDa schistosomulum surface antigen (GP 38) (47). This antigen is present on cercariae and schistosomula, but is no longer detectable at the lung stage of infection. On the other hand, this same monoclonal antibody is able to recognize a 115-kDa molecule in the metabolic products of *S. mansoni* adult worms. Cross-absorption experiments indicate the very likely existence of common epitopes on the 38-kDa and 115-kDa molecules, and immunization of rats with the 115-kDa antigen induces an antibody response against the GP 38 surface molecules (45). These give a molecular basis to the general concept of concomitant immunity, so far described on biological criteria. Conversely, a monoclonal antibody raised against adult worms could recognize a surface antigen on schistosomula (98). The particular antigenicity of the GP 38 antigen was also demonstrated by the observation that almost 100% of an infected human population developed an antibody response against this molecule, the response reaching a maximum during the later part of life, i.e. when epidemiological studies indicate the development of immunity (48).

The characterization of the target antigen of IgE antibody was made possible by the study of excretion-secretion products of schistosomula. In this material, two major antigens bind specific IgE antibodies. Immunization with schistosomulum-released products permitted the induction of a marked specific IgE response in rats, accompanied by a significant degree of protection in these animals. Passive serum transfer

reveals the IgE dependency of the protection and its association with a response to 22–26-kDa molecules (6).

The Application of Molecular Biology to Schistosomes

The recent, relatively massive application of gene-cloning techniques to the study of *Schistosoma* sp. has been concerned with two main objectives: (*a*) the in vitro synthesis of *Schistosoma* sp. antigens to identify and clone larval stage surface antigens, and (*b*) the use of direct genome analysis to identify and distinguish between schistosome species and subspecific variants, which are not discussed here.

The approach toward the eventual cloning of schistosomula surface antigens has consisted of the isolation of mRNA from different life-cycle stages of *S. mansoni*, their in vitro translation, essentially in the rabbit reticulocyte lysate system, and the immunoprecipitation of translated antigens by infection and immunization sera. The delicate stage is evidently the identification of surface antigens or their precursors among the translation products.

Adult worm RNA was the first isolated, and in the rabbit reticulocyte lysate translation systems it yielded at least 13 antigenic species immunoprecipitable by the serum from a infected monkey (102). Comparison of translation patterns obtained with RNA from cercariae, schistosomula, and adult worms showed that infected human serum immunoprecipitated molecules in the ranges 27–37, 46–63, and at 70 kDa, whereas rat serum immunoprecipitated proteins maximally in the 30–45-kDa range. Very similar patterns are obtained for the two larval stages, but adult worm RNA seemed to translate for proteins of lower molecular weight (52). Egg stage RNA has also been translated (38, 100) and the immunoprecipitation pattern obtained with sera from immunized rabbits was very similar, except that a prominent 40-kDa band was present in the egg RNA translation products.

We have recently studied the effect of protein maturation (79), using *Xenopus laevis* oocytes microinjected with adult worm RNA. In this system, *S. mansoni* antigens were processed, glycosylated, and segregated into different compartments. A multiple series of molecules in the 50–60-kDa range were N-glycosylated, segregated into the membrane fraction, and secreted from the oocytes; a major 29-kDa molecule was not glycosylated and could be detected only in the cytosol fractions. In this system, at least 20 different antigenic species could be immunoprecipitated by human infection sera.

The search for schistosomulum surface antigens amongst the translation products of different life-cycle stages has been pursued both directly and indirectly. To date, however, most attempts to immunoprecipitate trans-

lation products with sera from infected animals or human patients or monoclonal antibodies have failed (63, 79), although recently two protective mouse monoclonal antibodies that recognize translation products of 28 and 37 kDa were described (95).

Several authors used indirect methods to determine the presence of molecules carrying epitopes expressed at the larval surface among translation products. Serum adsorbed with live schistosomula identified two translation products at 15 and 22 kDa as putative schistosomulum surface antigens. Interestingly, these antigens were intensely synthesized by RNA from adult worms and sporocystes, but not from schistosomula. These antigens were equated with molecules of the same molecular weight detected at the surface of schistosomula after ^{125}I-lactoperoxidase labelling (63). It should be remembered, however, that epitopes expressed by the larval stages of *S. mansoni* are expressed on molecules of higher molecular weights by adult worms (45, 97). In addition, translation products in this range of molecular weight (15–22 kDa) are glycosylated in the *X. laevis* translation system, and it is thus dangerous to relate molecular weights for reticulocyte lysate translation products with those of native proteins.

Another indirect technique employed has been the use of fixed schistosomula as the solid immunoprecipitation support instead of Protein-A-Sepharose (100). In this way, only antibodies recognizing both the schistosomulum surface and translation products are immunoprecipitated. Using hyperimmune rabbit serum, workers found five components could be identified in rabbit reticulocyte lysate products at 24, 27, 28, 86, and 100 kDa.

Using *X. laevis* translation products and sera absorbed on live schistosomula or in competition with a schistosomulum tegument extract, we were able to define three components at 29, 43, and 100 kDa as putative surface antigens, with both rat and human sera (79). The present multiplicity of possibly interesting antigens as subjects for subsequent cloning experiments renders absolutely necessary the unequivocal identification (preferably using monospecific antibodies) of potentially protective antigens.

The only published example to date of cloning of cDNA corresponding to *S. mansoni* antigens concerns the cloning of cDNA corresponding to the 40-kDa major translation product of egg-stage RNA in a pBR325-based vector (38). The 40-kDa antigen would seem to be stage and species specific, and hence the cloned protein could be of use in immunodiagnosis.

REGULATION OF EFFECTOR MECHANISMS

Extensive studies concerning parasite survival in the immune host reveal their remarkable ingenuity in escaping the host immune response. Among

the numerous mechanisms that have been described, we discuss only those related to the regulation of effector cell function or to interfering with antibody recognition at the surface of schistosomula.

Parasite-Derived Inhibitory Molecules

The possibility that parasite molecules could act as inhibitors of various cell functions was suspected after the initial demonstration that metabolic products of *S. mansoni* adult worms could strongly inhibit lymphocyte proliferation in vitro and in vivo (19, 20, 40). This factor named SDIF (schistosome-derived inhibitory factor) was shown to be of low molecular weight, heat stable, and able to inhibit T-cell proliferation selectively. SDIF inhibits in particular the generation of CTL in mixed lymphocyte cultures without inhibiting IL_2 production (68). Purified preparations selectively inhibit the proliferation of IL_2-dependent as well as IL_2-independent T-cell lines. In contrast, macrophage and B-cell lines were not inhibited by purified SDIF.

Cytofluorographic studies showed that SDIF has no effect on cells in G_0 phase of the cell cycle. T cells progress through the G_1 phase, shown by the normal production of IL_2 and the unaltered expression of IL_2 receptors. The inhibitory effect of SDIF seems to be located in later phases of the cell cycle (Mazingue et al, manuscript in preparation).

Recent experiments showed that SDIF is able to diminish markedly the primary and secondary IgE response of rats immunized with DNP-OVA when it is injected either simultaneously or shortly after antigen administration (65). These effects could be attributed to the inhibitory effect on lymphocyte proliferation.

The specific inhibition of T-cell proliferation by SDIF indicates a possible use of this factor as an immunosuppressive agent. Furthermore, the extreme sensitivity of malignant T-cell lines to this factor could be of interest in the therapy of T-cell leukemia.

Macrophage Inhibitory Peptides

A possible regulatory mechanism of macrophage activation has also been demonstrated.

The identification of inhibitory peptides comes from convergent observations made on the existence of Fc receptors for IgG on the surface of schistosomula (103) and the demonstration of the release of proteolytic enzymes by the parasite at different life stages. Shortly after IgG molecules bind through their Fc portion to the membrane receptor on schistosomula, parasite proteases identified as a serine protease and an amidopeptidase were able to cleave the bound molecules and lead to the release of peptides (7, 10).

The precise role of these peptides on the regulation of effector functions of various cell populations was explored, and it was demonstrated that they significantly decreased macrophage activation, assessed by the release of lysosomal enzymes, glucosamine incorporation, superoxide anion generation, and phagocytosis. The production of Interleukin 1 was also inhibited. Furthermore, IgE-dependent macrophage cytotoxicity for schistosomula was dramatically decreased (10, 11).

A series of investigations recently allowed the characterization of the responsible peptide. The main inhibitory activity could be attributed to a tripeptide (ThrLysPro), which has been synthesized. The synthetic tripeptide reproduces at concentrations down to 10^{-12} M the biological effects of the IgG cleavage peptides (8, 9).

These two examples illustrate the potential interest of these regulatory molecules not only in the context of host parasite interplay, but also as antiinflammatory or immunosuppressive agents.

Regulation by Antibody

Another regulatory mechanism was shown to act at the recognition stage of the target antigen on the schistosomulum surface.

Rat monoclonal anti-*S. mansoni* antibodies of the IgG_{2c} isotype, specifically directed against the schistosomulum surface, did not exhibit any antiparasite killing effect in vitro. However, they were able to inhibit in a dose-dependent manner the cytotoxic effect mediated by IgG_{2a} and eosinophils. Immunoprecipitation and cross-absorption experiments indicated that this monoclonal was able to react with the 38-kDa glycoprotein defined by the protective IgG_{2a} antibody.

This inhibitory effect observed in vitro was confirmed in vivo by the observation that IgG_{2c} monoclonal antibody could strongly decrease the protective role of the IgG_{2a} monoclonal. This inhibition could also be achieved with $F(ab')_2$ fragments of the IgG_{2c} monoclonal, which indicates a possible blocking action on the recognition of the schistosomulum surface by IgG_{2a} antibody (54). The existence of such a mechanism raises the fascinating question of the existence of blocking antibody modulating the efficiency of immune effectors in schistosome infection.

Recent evidence has been obtained showing the existence of such antibodies in human infections. This leads to the concept that whereas potential markers of immunity in man, defined by protective monoclonal antibodies, might be present in many individuals, and this appears to be the case for the antibody response to GP 38, the clinical expression of immunity might be controlled by the presence or the absence of a blocking antibody response. In this respect, although it might first appear paradoxical, it could be more important in the future to try to characterize markers of nonimmunity rather than putative indicators of protection.

CONCLUDING REMARKS

Considered in the general framework of the immune response to pathogens, the study of effector mechanisms against schistosomes has been highly beneficial not only to parasite immunology but also to our general knowledge of the immune response and its regulation. At least, some general concepts may emerge from such studies:

1. The role in ADCC of so far unsuspected cell populations, leading recently to the identification of new functions for platelets;
2. The existence and biological significance of low-affinity receptors for IgE on a variety of cells;
3. The functional importance in protective immunity for parasites of IgE and anaphylactic antibodies in general;
4. The existence in biological models of a complex network of regulatory mechanisms interfering with effector cell activation or target recognition.

These comments might help us understand why the development of a vaccine against schistosomes, although feasible now that we have means to identify and clone functional antigens, may still be a long way ahead, but also how schistosomes have served immunology well.

ACKNOWLEDGMENTS

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 477–500

IMMUNOLOGIC ABNORMALITIES IN THE ACQUIRED IMMUNODEFICIENCY SYNDROME[1]

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INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) has become one of the most devastating diseases of the adult immune system ever described. The varied clinical manifestations of this disease, opportunistic infections, and neoplasms (particularly Kaposi's sarcoma), are indicative of an essentially nonfunctioning cellular host defense system (1–7). The diagnosis of AIDS is a clinical one, defined as the occurrence of an illness predictive of a defect in cell-mediated immunity occurring in an individual with no known cause for diminished resistance to that disease. Such illnesses include Kaposi's sarcoma in patients under the age of 60, *Pneumocystis carinii* pneumonia, and cytomegalovirus (CMV) retinitis (8). This strict clinical definition does not include the full spectrum of AIDS manifestations, however, which are thought to range from an asymptomatic state with laboratory evidence of immune deficiency to a variety of nonspecific syndromes such as the pre-AIDS syndrome, or the AIDS-related complex, characterized by fever, weight loss, and lymphadenopathy, to the frank AIDS as defined above.

A unique feature of this illness is that it is an epidemiologically restricted disorder with the majority (73%) of the cases occurring in homosexual or

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bisexual men. Other groups at risk for developing AIDS are intravenous drug abusers (17%), hemophiliacs, recipients of blood or blood products, heterosexual contacts of members of the above groups, and individuals of Haitian origin (8). Of note is the fact that seemingly healthy individuals within these risk groups may exhibit marked immunologic abnormalities without evidence of clinical illness (9–12).

One of the major difficulties in attempting to understand the nature of the immunologic abnormalities in AIDS is the fact that many of the infections that these patients develop are capable of inducing marked changes in immune profile. For this reason, many investigators feel that the most appropriate patients for immune system study are those who definitely have AIDS, yet are at the very earliest stages of the illness. These are usually patients with Kaposi's sarcoma alone and total lymphocyte counts above $1000/mm^3$.

The recent identification of a series of human T-cell tropic retroviruses known collectively as lymphadenopathy-associated virus (LAV) (13) or human T-cell leukemia/lymphoma virus (HTLV)-III (14) has provided evidence to support the hypothesis that this disease is due to an infectious agent specific for the helper/inducer T cell. The manifestations of immunologic abnormality in this syndrome are protean; and although the primary lesion is felt to be at the level of the helper/inducer T cell, abnormalities of immune function have been described at virtually all levels of the immune system including B-cell function, monocyte/macrophage function, and natural killer cell function. These abnormalities are summarized in Table 1.

ABNORMALITIES OF T LYMPHOCYTES

Quantitative Abnormalities of T Lymphocytes

The earliest descriptions of AIDS pointed out that patients with this syndrome manifested immune profiles characterized by a reversal in the ratio of helper/suppressor T cells (1, 3). Detailed study of this phenomenon has revealed that the peripheral blood lymphocytes of patients with AIDS are characterized by a marked decrease in the number of helper/inducer T cells and elevated, normal, or decreased numbers of suppressor/cytotoxic T cells (15–19).

A typical fluorescence-activated cell-sorter profile for a patient with AIDS and one for a healthy control are shown in Figure 1. As can be seen, the peripheral blood of the patient has very few cells bearing the T4 phenotype. Quantitation of the number of peripheral-blood lymphocytes bearing the T4 marker has revealed a heterogeneity among the clinical subgroups of AIDS and AIDS-related illnesses, with the highest total T4

Table 1 Immunologic abnormalities in AIDS

Quantitative abnormalities of T lymphocytes
Decreased numbers of helper-inducer (T4, Leu-3) cells
Elevated, normal, or decreased numbers of suppressor-cytotoxic (T8, Leu-2) cells
Functional abnormalities of T lymphocytes in vivo
Host susceptibility to infection
Host susceptibility to neoplasms
Decreased delayed-type hypersensitivity responses
Functional abnormalities of T lymphocytes in vitro
Elevated spontaneous proliferation
Decreased proliferative responses to mitogens and antigens
Decreased virus-specific cytotoxic lymphocyte function
Decreased ability to provide help to B lymphocytes
Functional abnormalities of B lymphocytes in vivo
Elevated serum immunoglobulin level
Circulating immune complexes
Inability to mount an appropriate serologic response following immunization
Functional abnormalities of B lymphocytes in vitro
Elevated spontaneous proliferation
Elevated numbers of spontaneous plaque-forming cells in the peripheral blood
Enhanced responsiveness to B cell growth factors
Refractoriness to the normal in vitro signals for B cell activation
Abnormalities of other cell types
Decreased monocyte/macrophage chemotaxis
Decreased natural killer cell activity
Abnormal suppressor phenomena in AIDS
Suppressor factors in serums
Antilymphocyte antibodies
T cell–derived suppressor substances
Miscellaneous serologic abnormalities
Elevations in acid-labile alpha interferon
Elevations in alpha-1 thymosin
Elevations in beta-2 microglobulin
Decreased serum thymulin levels

numbers among the patients with the lymphadenopathy syndrome and the lowest T4 numbers among the patients who had previously experienced opportunistic infections (Figure 2). This is the immunologic correlate of the clinical observation that patients with Kaposi's sarcoma alone have a longer life expectancy than patients who have had an opportunistic infection.

T4 cells can be further subdivided into helper and inducer subpopulations with the use of the monoclonal antibodies TQ-1 or Leu-8. The initial

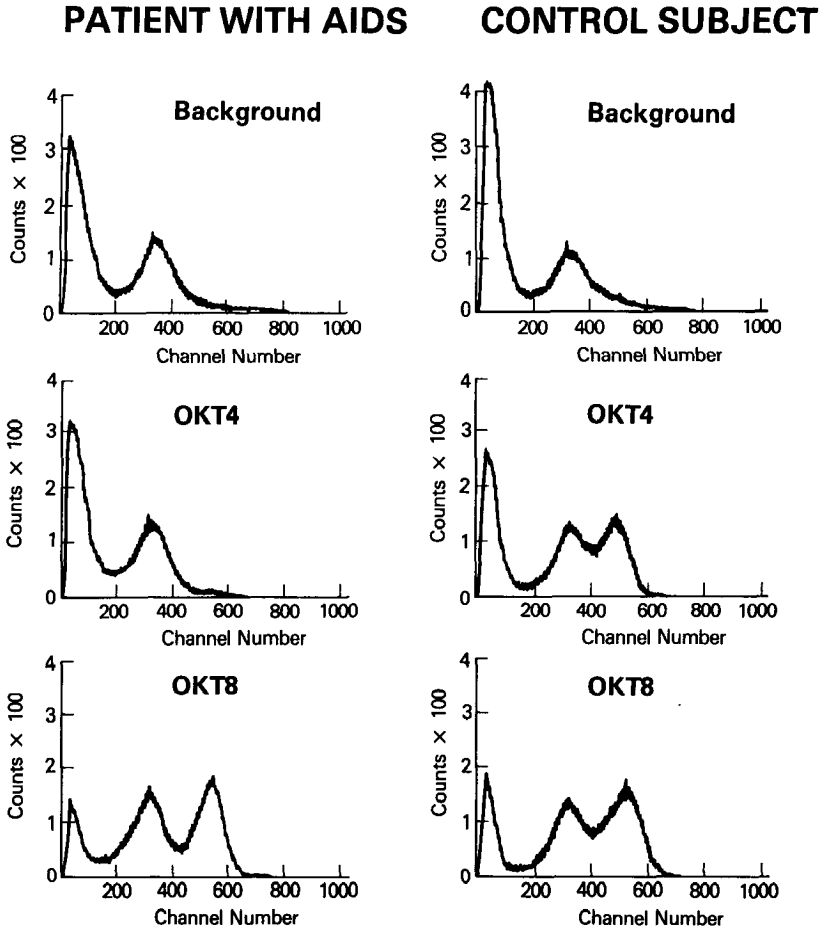

Figure 1 Nongated fluorescence-activated cell-sorter pattern of unfractionated peripheral blood mononuclear cells from an AIDS patient and his identical twin brother (taken from Ref. 7).

drop in T4 number, as judged by studies of pre-AIDS patients, appears to be due to a decrease in cells that express either Leu-8 or TQ-1 and thus are phenotypically inducer cells (20).

Total suppressor/cytotoxic cell numbers are generally elevated in homosexual men, patients with the pre-AIDS syndrome, and patients with only Kaposi's sarcoma. In contrast, suppressor/cytotoxic cell numbers are highly variable in patients who have had opportunistic infections (Figure 3). An elevation in T8 positive cells is often seen following viral infection with either Epstein-Barr virus (EBV) or CMV (21, 22). By virtue of the fact that more than 90% of patients with AIDS are actively infected with one or

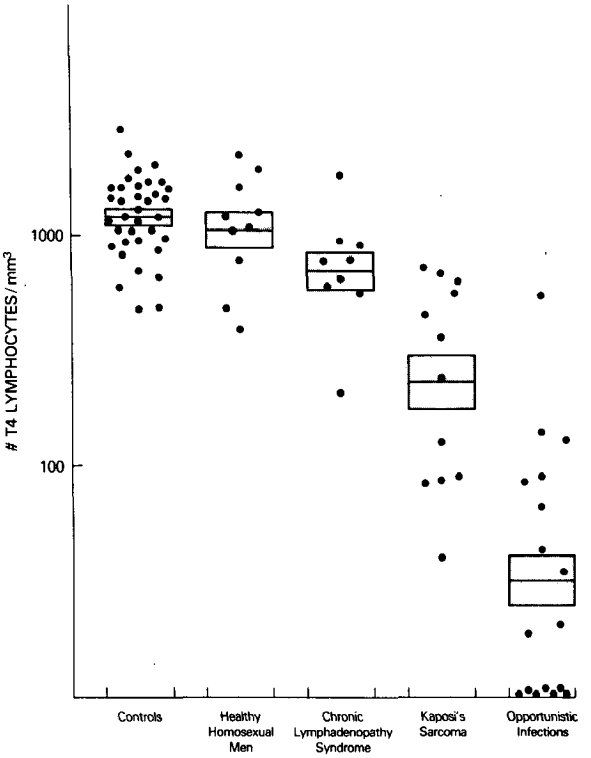

Figure 2 Total number of peripheral blood T4+ lymphocytes for the clinical subpopulations of AIDS patients, chronic lymphadenopathy syndrome patients, and controls (taken from Ref. 19).

more of these viruses (23), it is most likely that these elevations in T8 number are part of the usual host response to viral infection.

Functional Abnormalities of T Lymphocytes

The functional study of the human immune system is hampered by the fact that the only lymphoid organ easily accessible for study is the peripheral blood. This is a dynamic organ whose precise makeup at any single time is subject to a wide range of internal and external influences. Despite this limitation, much has been learned about the immune systems of AIDS patients through the study of peripheral-blood lymphocytes.

In vivo T-cell function can be measured by the ability of the host to mount a delayed-type hypersensitivity response. This complex immune function, requiring antigen processing, antigen-specific T cells, and non-specific recruitment of mononuclear cells, is markedly decreased in patients with AIDS (1–3, 5, 24, 25). Utilizing a panel of seven skin-test antigens, the

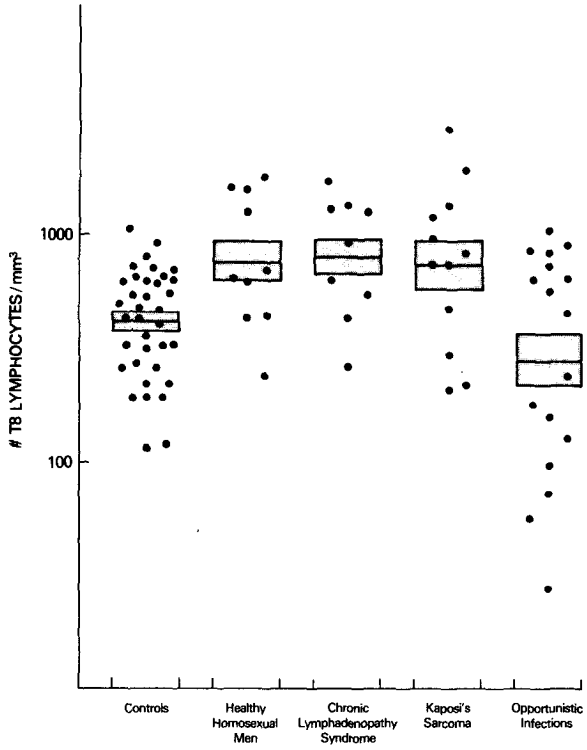

Figure 3 Total number of peripheral blood T8 + lymphocytes for the clinical subpopulations of AIDS patients, chronic lymphadenopathy syndrome patients, and controls (taken from Ref. 19).

positive responses of a group of AIDS patients were approximately 25% those of the control group (Table 2). Other studies using more advanced patients have reported a complete absence of skin-test reactivity in patients with AIDS.

A wide variety of in vitro studies have been performed on the peripheral-blood T lymphocytes of patients with AIDS. Among the abnormalities reported are elevated spontaneous proliferation, decreased mitogen-induced or antigen-induced proliferation, decreased cytotoxicity, and decreased ability to provide help to B lymphocytes. As is discussed below, some of these are due to alterations in lymphocyte subset numbers, some appear to be qualitative abnormalities of the subset, and some are related to the secondary infections that the patients develop.

Unfractionated, peripheral-blood T cells and isolated T-cell subsets have been examined for their ability to incorporate ^{3}H-thymidine spontaneously during the first 12 hours of culture. As shown in Table 3, both T4 and T8

Table 2 Skin testing reactivity among AIDS patients and controls

	Test antigen[b]							
	Control	Tetanus	Diphtheria	Strep	TB	Candida	Trichophyton	Proteus
Patients N = 20	0[a]	10	5	15	0	10	0	40
Controls N = 10	0	90	80	70	60	90	80	50

[a] Expressed as % positive = # positive/# tested.
[b] Skin tests were applied using the Merieux Multitest® apparatus.

Table 3 Spontaneous lymphocyte proliferation in AIDS

Cell type	AIDS	Control
Unfractionated	256±49[a]	54±12
T	327±26	55±16
T4	388±105	68±8
T8	278±45	76±36
B	1006±151	342±113

[a] Expressed as cpm ^{3}[H]thymidine incorporated during the first 12 hours of culture ±1 SEM.

populations display markedly enhanced uptake, indicative of a state of in vivo activation. This elevation is seen in some but not all AIDS patients, and therefore probably represents a secondary, rather than a primary, phenomenon.

Another indicator of in vivo T-cell activation is the percentage of cells bearing the surface markers HLA-Dr or T10 or receptors for interleukin-2 (IL-2). When two-color staining is used, enhanced expression of both HLA-Dr and T10 has been demonstrated on the peripheral-blood T8 cells from patients with AIDS and patients with the pre-AIDS syndrome. Although possible to detect following in vitro activation with mitogen, there is no evidence that AIDS T cells express the Tac antigen (IL-2 receptor) in vivo (15).

AIDS lymphocytes demonstrate decreased blast transformation in vitro to mitogens such as pokeweed mitogen (PWM) as well as antigens such as tetanus toxoid. The different clinical subgroups show a declining pattern of responsiveness similar to that of total T4 number for both responses. Although both PWM and tetanus toxoid responses are decreased in cultures of unfractionated cells, marked differences are seen between antigen- and mitogen-induced responses when one studies purified T-cell subsets (Table 4). In the case of the mitogen-induced responses, the decreased proliferation appears to be due solely to decreased numbers of T4 cells in culture. When purified T4 cells are studied, responses of AIDS cells are comparable to responses of normal cells. Therefore, this defect in mitogen responsiveness is not at the level of the single cell but at the level of the composition of the unseparated cell population. In contrast, in the antigen-induced cultures, proliferative responses of patients are markedly diminished in both unfractionated cells and purified T4 cell cultures. This inability of the T4 cell population to mount a proliferative response to soluble antigen appears to be one of the earliest qualitative defects in the immune systems of patients with AIDS (Table 4).

Table 4 Proliferative responses of AIDS or control lymphocytes to pokeweed mitogen or tetanus toxoid

Cell type	Pokeweed mitogen		Tetanus toxoid	
	AIDS	Control	AIDS	Control
Unfractionated	1400 ± 800[a]	10,800 ± 1900	< 100	20,300 ± 6400
T4	17,800 ± 2600	19,600 ± 2200	< 100	16,900 ± 1200
T8	3100 ± 515	4400 ± 680	< 100	4300 ± 600

[a] Expressed as cpm ± 1 SEM at the end of a five-day culture.

In addition to their decreased ability to mount a proliferative response to soluble antigen, AIDS mononuclear cells are essentially unable to mount a cytotoxic lymphocyte response against CMV-infected syngeneic target cells or against targets of natural killer cells (26). The inability to mount a CMV-specific cytotoxic lymphocyte response in vitro is correlated with poor outcome in CMV-infected bone marrow transplant recipients (27). The lack of this in vitro immune function in AIDS patients is probably reflected in the extremely high prevalence of active CMV infection in these patients and the frequency with which CMV is felt to be a major cause of morbidity and mortality (23, 28). Considering that this immune effector function is felt to be mediated by T8 cells and that T8 levels are generally normal in patients with AIDS (Figure 3), the most likely explanation for decreased CMV-specific cytotoxicity in these patients is lack of an effective inducer signal in vivo. This hypothesis is supported by the fact (discussed below) that CMV-specific cytotoxic lymphocytes appear to be present in adequate numbers in the peripheral blood of AIDS patients, as is evidenced by the fact that CMV-specific cytotoxicity can be demonstrated in vitro following incubation of the cells with IL-2 (26).

Unfractionated T cells from AIDS patients have been shown to be a poor source of help with respect to pokeweed mitogen (PWM)–induced B-cell differentiation (5, 29, 30). In experiments designed to correct for alterations in lymphocyte subset numbers, this decreased ability to provide help to B lymphocytes was shown to be due to decreased helper activity of the T4 cells, rather than increased suppression by the T8 cells (29, 30). This functional abnormality is somewhat variable and may be close to normal in patients with recent onset of disease. Thus, it is unclear at present whether this is a primary defect in AIDS or secondary to infection with an opportunistic virus.

AIDS patients have been noted to have decreased reactivity in both allogeneic– and autologous–mixed lymphocyte cultures (31). The decrease in allogeneic reactivity has been shown to be due to a quantitative decrease

in the T4 lymphocytes in the peripheral blood, while the decrease in autologous reactivity appears to be a qualitative defect in the Leu-3 (T4) subpopulation that is not corrected by adding an increased number of Leu-3 cells to culture. This defect in autologous reactivity has also been described in individuals at risk for AIDS, and correlates with the fact that both responses to soluble antigen and autologous reactivity are felt to residue within the same T-cell subpopulation (32).

IL-2 in AIDS

The observations of decreased mitogen responsiveness in AIDS stimulated several groups to investigate whether or not there were abnormalities in the IL-2 systems in these patients. Initial studies examining the supernatants of PHA-, concanavalin A–, or tetanus toxoid–stimulated cultures, revealed a decrease in IL-2 production (33) accompanied by decreased proliferative responses as noted above. However, not all studies confirmed these observations of decreased IL-2 production (34, 35). This discrepancy was resolved by studies using purified T-cell subsets which revealed that either purified T4 or purified T8 cells from AIDS patients were capable of producing, in vitro, levels of IL-2 comparable to those of controls. Similar results were obtained in studying the expression of IL-2 receptors in the form of the Tac antigen on lymphocytes from patients with AIDS. While unfractionated cells were noted to be deficient in IL-2 receptor expression following mitogen stimulation (35), purified T4 and T8 subsets expressed normal amounts of Tac antigen.

In addition to attempting to define defects in the IL-2 system in AIDS, a variety of studies have examined the ability of purified IL-2, either natural or recombinant products, to modulate in vitro immune responses of AIDS lymphocytes (33, 36). While most studies have revealed enhanced proliferative responses in mitogen-stimualted cultures upon the addition of IL-2, it is unclear whether there is actually enhanced responsiveness to mitogen or merely an additive effect of mitogen-induced and IL-2-induced proliferation. As shown in Table 5, IL-2 is capable of inducing substantial proliferation in unstimulated human peripheral-blood mononuclear cells from either AIDS patients or control individuals in the absence of an activation signal. In these experiments IL-2 appeared to increase proliferation as an additive rather than a synergistic factor.

IL-2 in vitro is capable of markedly enhancing both CMV-specific cytotoxicity and natural killer cell activity of either AIDS or control mononuclear cells. This phenomenon is seen following brief incubation with IL-2 and is not mediated through gamma interferon release (26, 37). Data such as these provide firm evidence in support of the hypothesis that

Table 5 Proliferative responses of AIDS or control unfractionated lymphocytes to interleukin-2 with or without a mitogenic signal

Stimulus	AIDS	Control
Control	470 ± 70[a]	381 ± 123
Pokeweed mitogen (1:200)	7678 ± 2749	29,874 ± 6816
Interleukin-2 (10 units)	3240 ± 1746	2892 ± 612
Pokeweed mitogen and interleukin-2	9396 ± 2578	23,920 ± 4360

[a] Expressed as cpm ± 1 SEM at the end of a 5-day culture.

the nature of the immune defect in AIDS is a lack of helper/inducer function rather than an excess of suppression.

B-Cell Abnormalities in AIDS

The demonstration that AIDS patients have normal or elevated serum immunoglobulin (Ig) levels was felt initially to represent the fact that humoral immunity is spared in this syndrome (1–4, 17). Shortly after the initial reports of AIDS, it became apparent that despite the normal quantities of Ig in the blood the B cells of these patients were characterized by a polyclonal activation as intense as that seen in any disease state characterized by hyperactive B-cell responses, including the prototype disease for polyclonal B-cell activation—systemic lupus erythematosus. This polyclonal B-cell activation has been extensively characterized and is manifested by elevated levels of Ig and circulating immune complexes (38), an inability to mount an antigen-specific antibody response following immunization (29, 39), elevated numbers of spontaneous Ig-secreting cells in the peripheral blood (29), enhanced responsiveness to B-cell growth factors, and refractoriness to the normal in vitro signals for B-cell activation.

Total IgG and total IgA levels are elevated in patients with AIDS. Despite this quantitative finding, the humoral immune systems of these patients are qualitatively unable to mount an appropriate serologic response following immunization. This is true following immunization with a potent primary protein antigen, such as keyhole limpet hemocyanin; a recall protein antigen such as tetanus toxoid; or a T cell–independent antigen such as pneumococcal polysaccharide (29, 39).

The normal scheme of B-cell activation involves discrete steps of activation, proliferation, and differentiation. These steps can be dissected in vitro through measurements of spontaneous and inducible B-cell activity. The peripheral blood of patients with AIDS has been found to contain a

markedly increased number of fully activated and differentiated cells, as evidenced by the number of cells spontaneously secreting Ig in a reverse hemolytic plaque-forming cell assay. This level of spontaneous plaque-forming cells is as high as has been described in any disease state (29).

In addition to increased numbers of B cells spontaneously secreting Ig, the peripheral blood of AIDS patients contains increased numbers of spontaneously proliferating cells and increased numbers of cells that will proliferate to a B-cell growth factor derived from a T-T hybridoma without the need for an in vitro activation signal. These phenomena are similar to what one may see in vitro following transformation with EBV.

B cells from AIDS patients are relatively unresponsive in vitro to standard pure B-cell activation signals, presumably due to the fact that the B-cell repertoire of these patients is already in a state of polyclonal activation. These include responses to formalinized whole *Staphylococcus aureus* Cowan strain I and polyvalent goat antihuman IgM (24). This refractoriness to activation signals in vitro probably corresponds to the refractoriness of the humoral immune system in vivo with respect to the ability to mount a new serologic response following immunization.

While the precise mechanisms underlying the polyclonal B-cell activation in AIDS remain to be determined, the most likely hypothesis seems to be the viral transformation of B cells in the absence of the normal regulatory T-cell environment. EBV as well as CMV can be isolated from virtually all patients with AIDS (23). Both of these DNA viruses of the herpes virus family are capable of inducing B-cell activation. While the degree of activation usually seen following in vivo infection in non-AIDS patients does not approach the magnitude seen in AIDS patients, the lack of a normal regulatory T-cell environment may allow this type of B-cell activation to proceed unchecked. Even though adequate numbers of suppressor cells are present in AIDS patients, they probably are unable to exert their normal effector function in the absence of an intact inducer-cell population. An alternative hypothesis to explain the B-cell activation in AIDS is that the T cells from these patients, as a result of infection with retrovirus, are constitutively secreting B-cell activating factors, as has been reported in the case of HTLV I–infected T cells (40). Further studies are required to determine the validity of this second hypothesis.

Abnormalities of Monocytes/Macrophages in AIDS

The number of monocytes in the peripheral blood of AIDS patients, as determined by nonspecific esterase staining, is generally normal; however, the monocytes are functionally abnormal in that they manifest a marked defect in chemotaxis (41). This defect was demonstrated over a wide range of concentrations of four potent chemotactic stimuli: N-formylmethionyl-

leucylphenylalanine; lymphocyte-derived chemotactic factor; C5a; and the soluble products of *Giardia lamblia.* The defect was most pronounced in patients at the latter stages of illness; it did not appear to be due to suppressor factors in the serums of patients. Whether this defect in chemotaxis is a primary or secondary feature of the immunologic abnormalities in AIDS is unclear.

Although defective in chemotactic ability, AIDS monocytes have been reported to be normal with respect to phagocytosis and intracellular killing of microorganisms. In response to gamma interferon, AIDS monocytes demonstrate normal enhancement of H_2O_2 release and cytotoxicity (42).

In vivo monocyte/macrophage function can be evaluated as part of the reticuloendothelial system through the use of red blood cell (RBC)-clearance studies. In these studies, autologous ^{51}Cr-labeled Rh^+ RBC are coated with anti-Rh antibodies and then reinjected into the patient. The rate of removal of the tagged RBC is then measured. Patients with AIDS have been shown to have a markedly decreased rate of removal of these coated RBC, implying a defect in Fc receptor–mediated clearance (43). This finding is most striking in patients with the most advanced disease and may be related to the blockage of Fc receptors in these patients by circulating immune complexes.

In addition to functioning as a component of the reticuloendothelial system, monocytes play an important role in cellular immune responses. As part of the cellular immune system, they are important accessory cells involved in the processing and presentation of antigen to immune-component cells. These latter functions involve the expression of class II major histocompatibility complex antigens and secretion of IL-1. Although epidermal Langerhans' cells from patients with AIDS are reportedly deficient in Ia expression (44), little definitive work exists that directly addresses the immunologic competence of monocytes from AIDS patients.

Abnormal Suppressor Phenomena in AIDS

The majority of the discussion thus far has centered on the proposition that the immunoregulatory abnormalities in AIDS result from a lack of helper/inducer function, particularly at the level of the antigen-specific T4 cell. In view of the fact that a hyporeactive immune system can be due either to a lack of inductive/helper influences or to an excess of suppressor influences, a great deal of work has been performed attempting to delineate possible suppressor mechanisms operative in patients with AIDS. Among the abnormalities that have been described are the presence of soluble suppressor factors in serum, the presence of antilymphocyte antibodies in serum, and the production in vitro of T cell–derived suppressor substances.

The serums from patients with AIDS have been described as containing

substance(s) capable of: (*a*) inhibiting alpha interferon–induced enhancement of natural killer cell activity of normal mononuclear cells, (*b*) causing a decrease in the mixed lymphocyte culture reactivity of normal lymphocytes, and (*c*) diminishing the PHA response of normal lymphocytes (45, 46). These inhibitory influences are most pronounced in the serums of the most seriously ill patients and appear to be related to the suppression of IL-2 production. This factor is not Ig, is not inactivated by 2-mercaptoethanol, and is not ether soluble. It can be removed by sheep RBC absorption but not allogeneic mononuclear cells absorption. The substance is capable of being inactivated when heated to 100°C for 10 minutes. The fact that it is not invariably present, coupled with its presence in highest titers in the most severely ill patients, suggests strongly that the substance reflects a secondary phenomenon rather than the primary immunologic defect in AIDS.

Antilymphocyte antibodies have been reported present in the serums of 61% of AIDS and pre-AIDS patients (47). These antibodies are heterogeneous in that some patients were reported to have antibodies directed toward T4 cells, while others had antibodies directed towards T8 cells. There was no correlation between the presence or type of antilymphocyte antibodies and the clinical or immunologic features of the patients. Most likely, this represents a secondary feature of the immune deficiency of AIDS and in fact may be reflective of the nonspecific polyclonal B-cell activation described earlier.

The interaction between T cells and adherent cells from AIDS patients has been shown to result in the generation of a potent suppressor substance capable of inhibiting spontaneous and PWM-induced Ig production and T-cell blast transformation to antigen (48). Monocyte bactericidal function and natural killer cell activity are unaffected by this substance. The substance has been found in supernatants of unstimulated peripheral-blood mononuclear cells from AIDS patients and as a product of a T-T hybridoma formed between T cells from an AIDS patient and a hypoxanthine/aminopterin/thymidine-sensitive human T-cell line (49). The factor acts during the first 48 hours of a T cell–dependent B-cell response and has no effect on the T cell–independent B-cell activation induced by EBV. It has a molecular weight of 47,000 and can be inactivated by 2-mercaptoethanol. In many respects, the factor is similar to the T cell–derived, soluble, immune repressor substance or concanavalin A–induced suppressor factors (50), and may reflect the enhanced number of activated T8 cells present in the peripheral blood of patients with AIDS.

As mentioned earlier, the peripheral blood of AIDS patients is characterized by an increase in the number of suppressor/cytotoxic lymphocytes, and phenotypically these cells bear increased amounts of HLA-Dr and T10

antigens. These antigens are found on mature lymphocytes in vitro following activation with antigen or mitogen or in vivo following infection with CMV or EBV. Despite this phenotypic evidence of in vivo activation of suppressor cells, there is little in vitro data to support the notion that these activated, phenotypically suppressor/cytotoxic cells are exerting a suppressor influence on the remainder of the immune system. The addition of mononuclear cells or purified subsets of mononuclear cells from AIDS patients to a variety of in vitro systems has consistently failed to reveal enhanced suppressor activity of the AIDS mononuclear cells in experiments where appropriate corrections were made for differences in percentage of T8+ cells in the preparations. An example of one such set of experiments is shown in Table 6; a variety of lymphoid populations were added to different indicator systems, either unfractionated cells from a normal individual or T4 cells from an AIDS patient. As can be seen, no abnormal suppressor function was present with respect to lymphocyte blast transformation in any of the cell populations from the AIDS patient. In addition, as indicated above, T8+ lymphocytes from AIDS patients were found to suppress an allogeneic system of PWM-driven Ig production to the same degree as did T8+ cells from a control individual (29).

Thus, while T8+ cells from AIDS patients exhibit phenotypic evidence of enhanced activation, they show little functional evidence of enhanced activation with the exception of elevated spontaneous prolifera-

Table 6 Lack of excess suppression among subpopulations of AIDS lymphocytes

	Test population			
	Control		AIDS	
Indicator population	Cell type[b]	Phytohemagglutinin[c] response	Cell type	Phytohemagglutinin response
Unfractionated control	—	22,100	—	22,100
	T4↓[a]	27,000	T4↓	21,200
	T8	33,000	T8	26,000
	$T4^-/T8^-$	18,100	$T4^-/T8^-$	19,700
	B+M0	45,400	B+M0	
T4 AIDS	—	17,000	—	17,000
	T4↓	15,200	T4↓	23,300
	T8	28,800	T8	26,100
	$T4^-/T8^-$	18,400	$T4^-/T8^-$	27,500
	B+M0	28,100	B+M0	36,300

[a] Cells received 1500 R prior to culture.
[b] 50,000 test cells added to 50,000 indicator cells.
[c] [^{3}H]thymidine incorporation measured on day 3.

tion (Table 3). In data not shown, T8+ cells from AIDS patients did not demonstrate enhanced in vitro responsiveness to IL-2. It is highly likely that the phenotypic expression of T8 activation is part of the physiologic response to viral infection and also likely that this activation is being aborted prior to differentiation in the case of the patient with AIDS, owing to the lack of an effective helper/inducer population.

Serologic Markers of Altered Immune Reactivity

A variety of serologic findings reflective of disordered immunoregulation have been reported to occur in the serums of patients with AIDS. Among these are elevations in acid-labile interferon (51), elevated levels of immune complexes (1, 38), elevations in alpha-1-thymosin (52), decreased levels of thymulin (53), and elevated levels of beta-2-microglobulin (54, 55). These abnormalities, while present in the majority of the patients with AIDS, are also found to a variable degree in many healthy risk-group members. They most likely reflect, rather than directly relate to, the primary immune defect in this illness. Their value as potential serologic screening tests for AIDS is currently under investigation. The recent association of AIDS with retrovirus infection and the development of reliable screening tests for antibodies to these retroviruses have decreased the potential utility of these other serologic measurements.

One of the earliest serologic abnormalities reported to occur in AIDS was an elevation in the level of acid-labile interferon. This unique form of alpha-interferon was initially described as being present in the serums of patients with systemic lupus erythematosus; there is evidence to suggest that it may be produced by B lymphocytes that have been infected with retrovirus (56). Elevations in acid-labile alpha-interferon were reported to be present not only in patients with AIDS but also in patients with pre-AIDS conditions and in some healthy homosexual men. Of note is the fact that levels appear to be the highest in patients with the most severely compromised immune systems. It seems most likely that this alteration is a result of, rather than the cause of, the underlying immune deficiency in AIDS.

A great deal of interest has centered around the role of the thymus in AIDS. Early histologic descriptions indicated that at autopsy thymus glands from AIDS patients revealed a severe involution with thymocyte depletion, absence of a definite cortex or medulla, and lack of Hassall's corpuscles, all suggestive of autoimmune or viral attack (57). In contrast to children with immunodeficiencies, patients with AIDS have been reported to have elevated alpha-1 thymosin levels. Similar elevations have been seen in healthy homosexual men. Alpha-1 thymosin induces T-cell maturation,

and it has been postulated that the elevated levels of this hormone in patients with AIDS and AIDS-related illnesses may reflect a physiologic response to an end-organ failure at the level of the mature helper/inducer T cell. However, the lack of correlation between serum levels of alpha-1 thymosin and total helper cell numbers is inconsistent with this hypothesis. Another hypothesis proposed is that the alpha-1 thymosin produced in these patients is biologically ineffective and that the increased levels represent an attempt at compensation. While the precise mechanism underlying the thymic abnormalities in AIDS remains to be delineated, this marker will probably not be a useful serologic test for AIDS.

As mentioned earlier, the peripheral-blood B-cell repertoire of patients with AIDS is characterized by an intense polyclonal activation. One result of this is elevated levels of circulating immune complexes. These immune complexes are predominantly IgG and IgA (38), and they may be responsible for the decreased Fc receptor clearance observed in patients with AIDS. While most likely a secondary immunologic event, this elevation in immune complexes may play an important clinical role in these patients. High levels of circulating immune complexes may be responsible for some of the clinical syndromes seen in AIDS such as immune thrombocytopenic purpura and nephrotic syndrome. It is of substantial interest that many of the immunologic features of AIDS are also seen in patients with systemic lupus erythematosus. These similarities suggest that further work is warranted concerning the possibility that certain forms of autoimmune disease may be related to infection with retroviruses.

Beta-2 microglobulin is a low molecular weight polypeptide that forms the light chain of the cell-surface class I–HLA alloantigen. This structure is present on the surface of all nucleated cells. An increase in serum level of beta-2-microglobulin is felt to be associated with an increased rate of cell-membrane turnover. Elevated levels of beta-2 microglobulin have been reported in the urine and serums of patients with AIDS and pre-AIDS syndromes and in the serums of healthy homosexual men. Increasing levels of this polypeptide may be one of the earliest signs that a patient is developing an AIDS illness. However, further work is needed to substantiate these claims.

The Role of Retrovirus Infection in AIDS

A novel group of human T cell–tropic retroviruses referred to as either LAV or HTLV-III have been consistently isolated from the peripheral-blood mononuclear cells of patients with AIDS and pre-AIDS conditions (13, 14). In addition, the serums of virtually all patients with AIDS and substantial numbers of risk-group individuals have been shown to contain antibodies

to these viruses (58). Of note is the fact that evidence of infection with this virus is exceedingly rare outside of the standard AIDS risk groups (homosexual men, intravenous drug abusers, and hemophiliacs).

In vitro inoculation studies have revealed that these viruses have a selective tropism for the T4 population of human peripheral-blood mononuclear cells and are cytopathic for these cells. In vitro isolation studies utilizing peripheral-blood lymphocytes from AIDS patients or individuals at risk for AIDS have revealed that in vivo the virus is predominantly contained within the T4 lymphocyte compartment (59).

In addition to the above data, LAV has been introduced into a variety of B-cell lines, the majority of which are transformed with EBV (60). Although able to infect B cells, whether or not these viruses are able to directly modulate B-cell function is still unclear.

The highly restricted epidemiologic nature of this disorder suggests that some preexisting host-susceptibility factor may have to exist before clinical disease due to infection with these retroviruses can occur. Some susceptibility factors that have been proposed include allogeneic suppression due to the entry of allogeneic lymphocytes into the circulation, infection with CMV, and drug abuse. These susceptibility factors may correspond to the fact that in vitro cell transformation with HTLV-III is much easier when the lymphocytes are in a state of activation.

Thus, the most likely hypothesis for the development of the immunologic abnormalities associated with AIDS appears to be that infection with the AIDS-associated retrovirus is followed by its dissemination throughout the antigen-specific subpopulation of helper/inducer T4 cells. As a result of infection with this cytopathic virus, the T4 subpopulation of lymphocytes is attacked and destroyed. This initial immunologic insult results in a critical blow to the immune system, which is then unable to function appropriately due to the lack of this essential subset.

The direct infection and subsequent elimination of the antigen-specific T4 cell subpopulation explains the profound defect in antigen-reactivity, which is so characteristic of AIDS, either in the form of absent delayed-hypersensitivity skin-test reactivity or a decreased in vitro lymphocyte blast transformation to soluble antigen. Owing to the absence of helper/inducer cells, a variety of additional immunologic abnormalities consequently develop. The host is unable to mount an antigen-specific response to a protein antigen; specific cytotoxic T cells cannot be generated; and T8+ cells cannot be induced to exert their suppressor influence upon Ig production. Thus, the cellular immune system is essentially dormant, with some of the cellular elements intact but unable to be induced. These alterations in the immune system, directly attributable to infection with retrovirus lead to a variety of secondary manifestations of immune

dysfunction, many of which result from the incomplete response of the host to the varied opportunistic infections that develop, particularly CMV and EBV. These include a wide range of serologic abnormalities, decreased monocyte/macrophage function, alterations in T8 phenotype expression, and polyclonal B-cell activation. Currently it is impossible to say accurately which of these immunologic changes are direct or indirect effects of infection with LAV or HTLV-III.

Attempts at Immunologic Reconstitution in Patients with AIDS

The potential immunologic approaches to the therapy of AIDS are quite varied. One could try to eliminate the causative agent, replace the injured cells, or attempt to bolster the immune function of the remaining immune elements. The recent isolation of the causative agent of AIDS has resulted in a full-scale effort to identify agents which can inhibit in vitro the replication of retrovirus. Since the in vitro replication of this virus is dependent upon the activity of the enzyme reverse transcriptase, the agents that inhibit this enzyme are attractive candidates. At present, one such agent receiving close attention is suramin (a drug used in the treatment of trypanosomiasis and onchocerciasis) (61). Clinical trials are currently being initiated with these agents. It is important to point out that the failure of other approaches to therapy thus far seems to indicate that unless the causative agent of this illness can be effectively eliminated, little can be done to reconstitute the immune systems of these patients.

Several centers have attempted to reestablish the immune systems of patients with AIDS through bone marrow transplantation or the adoptive transfer of lymphocytes (62–64). This approach, while quite effective in the treatment of certain congenital immunodeficiencies, has been uniformly unsuccessful in the treatment of patients with AIDS. Transplants to both conditioned and unconditioned recipients have failed. In one series of experiments combining bone marrow transplantation with the adoptive transfer of lymphocytes utilizing a pair of identical twins, clear immunologic improvement was documented in the patient in the form of increased numbers of T4 cells and the development of a delayed-type hypersensitivity reaction to keyhole limpet hemocyanin. These immunologic changes, which developed over a period of several months and seemed to indicate a successful transplant, were only transient (Figure 4). The temporary nature of the improvement was strong evidence that persistence of the causative agent of AIDS was a major problem in utilizing this form of therapy.

As noted above, several agents, particularly IL-2 and gamma-interferon, have been shown to be capable of enhancing the immunologic function of

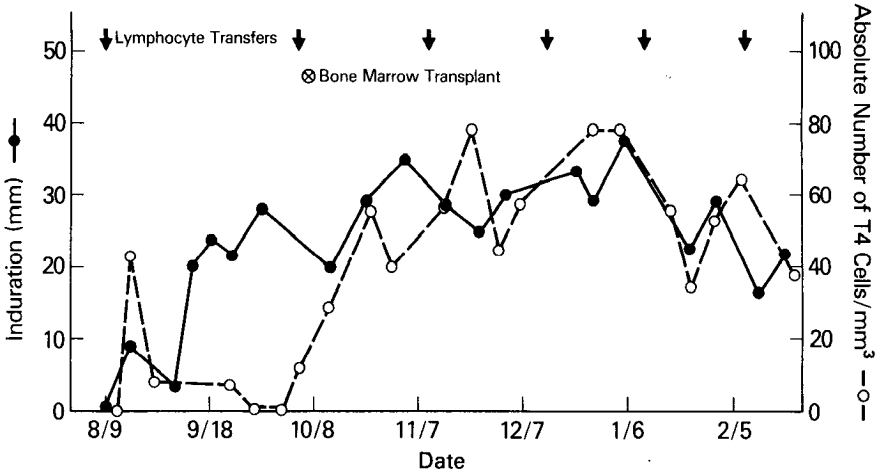

Figure 4 Total number of T4+ lymphocytes and degree of delayed-type hypersensitivity reaction to keyhole limpet hemocyanin in a patient with AIDS as a function of time following attempts at immune reconstitution. T4 number is represented by the open circles and skin test reactivity by the closed circles. The timing of lymphocyte transfers and bone marrow transplantation are given (taken from Ref. 62).

AIDS mononuclear cells in vitro. In the case of IL-2 this improvement was in the form of enhanced natural killer cell activity and improved CMV-specific cytotoxicity, while gamma-interferon was shown capable of enhancing monocyte-mediated cytotoxicity.

Clinical trials of both natural product and recombinant IL-2 are currently under way. Protocols employing the in vivo administration of gamma-interferon are currently being initiated. In contrast to the in vitro data, in vivo administration of IL-2 at doses capable of providing sustained serum levels of at least 4 units/ml for 5 continuous days was unable to cause any significant changes in cytotoxicity (65). This did not appear to be due to a peripheral margination of cytotoxic cells because in vitro boosting with IL-2 still was capable of inducing normal levels of cytotoxicity. Nonetheless, significant immunologic changes were noted in the patients receiving IL-2; these changes took the form of increased delayed–cutaneous hypersensitivity reactions; decreased total peripheral lymphocyte counts (predominantly due to a lowering of T8 numbers); and decreased serum Ig levels. One possible hypothesis for the decreased T8 numbers and the decreased Ig levels is that IL-2 was capable of inducing the differentiation of T8 cells into potent suppressors of Ig production. Despite these immunologic changes, no clinical improvements were seen in the patients. Further studies are required to assess accurately the potential use of this lymphokine in the therapy of AIDS.

SUMMARY

The immune systems of patients with AIDS are characterized by a profound defect in cell-mediated immunity which is predominantly due to a decrease in the number and function of the helper/inducer T lymphocytes, particularly the antigen-reactive cells. This defect is manifested primarily as decreases in delayed-type hypersensitivity reactions and decreased in vitro proliferation to soluble antigen. A variety of secondary manifestations of immunologic dysfunction occur, some of which result from a lack of effective inducer-cell function, others from the occurrence of opportunistic infections. Among these secondary phenomena are decreased cytotoxic lymphocyte responses, polyclonal B-cell activation, decreased monocyte chemotaxis, and a number of serologic abnormalities.

The primary cause of this defect in the antigen-reactive helper/inducer T lymphocyte is infection with a class of T-cell tropic retroviruses known as HTLV-III or LAV. This virus is capable of selectively infecting T4+ lymphocytes and can be isolated consistently from patients with AIDS or AIDS-related conditions.

Despite substantial knowledge concerning the nature of the immune defect in AIDS and its causative agent, little progress has been made in developing effective therapies for this uniformly fatal illness. Because the incidence of this disease continues to increase, and patients striken with this illness have a median survival of two years, additional investigation in this area is greatly needed. Continued effort aimed at delineating the precise nature of the immune defect in these patients should be of value in attempting to enhance our understanding of the human immune system in both normal and disease states.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 501–35

THE ATOMIC MOBILITY COMPONENT OF PROTEIN ANTIGENICITY

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INTRODUCTION

A full understanding of the cellular immune response requires a detailed knowledge of the structural basis for protein antigenicity at a molecular level that approaches atomic resolution. The development of techniques using anti-peptide antibodies (1, 2) as well as the continued growth of information derived using monoclonal antibodies to whole proteins (3) makes it possible to examine the antigenicity of specific protein sites. As a result, recent studies, using both anti-peptide antibodies corresponding to protein sites and monoclonal antibodies that are directed against proteins of known three-dimensional structure have produced new information concerning the relationship between antigenicity and protein structure.

In the simplest sense, antigenic determinants or epitopes of proteins represent those amino acid residues that interact with the binding site of an antibody molecule. It is now apparent that the set of antigenic specificities seen after immunization with the native protein is not representative of the entire potential antigenic repertoire. Yet, certain sequences and residues have been identified as immunodominant sites in that they represent epitopes to which most of the immune response is directed. Although the exact mechanism is unknown, immunodominance has been attributed to either intrinsic structural properties of the antigen or host regulatory mechanisms including tolerance, immune gene response, and specificity of T-cell help. While it is likely that both intrinsic and regulatory mechanisms

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are involved, this review focuses on the structural property of local flexibility or site mobility and its role in antigenicity.

A number of parameters have been suggested as being influential in protein antigenicity including surface exposure (4, 5), hydrophilicity, and residue type (6). Although antibodies have been assumed to recognize structural features exposed at the protein surface (see review 3), some epitopes are at least partially buried in the native protein structure (7) suggesting that antigenic recognition must sometimes involve structural rearrangements of the antigen. In fact, the role of segmental or domain flexibility in antigen-antibody interaction has long been appreciated; immunoglobulins are known to be composed of structurally separated but covalently linked domains allowing the binding sites to move while staying joined. More recently, the development of techniques to produce antibodies against synthetic peptides that also react with the intact protein (1) led to the proposal of several possible structural models for antigenic recognition (8). One of these models suggested that the peptides that elicit antibodies recognizing the intact protein might correspond to locations of relatively high local mobility in that protein. Not only does this model now appear to be correct, but the local flexibility proposed to explain the reactivity of anti-peptide antibodies is now implicated as a parameter in the antigenicity of intact proteins.

The major difficulty in correlating specific structural parameters with antigenicity has been the lack of complete antigenic data for a sufficient number of known protein structures. In this regard, the growth of communication between structural biochemists and immunologists promises to provide a great deal of new information. On those proteins for which the epitopes are known, there is still the basic problem of negative results: i.e. the inability to raise a monoclonal antibody to a given protein site does not prove that the site is nonantigenic.

In examining the molecular basis of antigenicity for antibodies raised against whole proteins, it is important to distinguish two types of epitopes. Discontiguous or conformational epitopes are formed by residues that are not contiguous in the sequence but are local in space due to the three-dimensional fold. Contiguous epitopes are formed by a sequence-continuous stretch of amino acid residues. The role of structural mobility in conformational determinants is difficult to define since they involve sequence-distant residues that may differ considerably in relative mobility. The role of mobility in the stereochemical complementarity of an antigen-antibody complex for a conformational determinant might well involve the shifting of only some of the residues, with a resultant change in the surface aspect and exposure of their neighbors. For contiguous determinants, the interpretation of site mobility is much less ambiguous and can often be

identified from a linear plot of the conformational mobility along the polypeptide chain. In addition, the ability to design anti-peptide antibodies allows mobility for contiguous determinants to be distinguished from the related parameters of exposed area and hydrophobicity somewhat more precisely than for conformational determinants in intact proteins. Unfortunately, this useful distinction between conformational and contiguous determinants can be difficult to make in practice. Often it is not known if all residues in a so-called contiguous determinant are involved in the epitope or conversely, whether a conformational determinant may have a contiguous component. Here we summarize information about local flexibility and antigenicity in proteins using the available structural and immunological data and then report the results of studies correlating antigenicity with mobility based upon two approaches: (*a*) dissecting a model protein by examining reactivity of its sites with representative anti-peptide antibodies, and (*b*) mapping the site specificity of antibodies to known protein structures.

DIFFERENTIAL MOBILITY OF PROTEIN SITES

In this review, we will address the role of atomic mobility in the antigenicity and immunogenicity of proteins. Before beginning, however, it may be helpful to review some general aspects of flexibility and mobility in proteins. The structural basis for protein flexibility is becoming increasingly clear from experimental evidence resulting from a variety of techniques. The dynamic properties of proteins are being characterized by the combination of single crystal diffraction with spectroscopic techniques such as NMR, ESR, fluorescence depolarization, perturbed angular spectroscopy, Mossbauer spectroscopy and hydrogen-exchange studies. One advantage of X-ray and neutron diffraction is the relatively high resolution available that provides specific information (in the atomic temperature factors) about the flexibility of individual amino acid residues and structural elements (for review, see 9). In addition, the location of atomic positions in proteins by single crystal diffraction has provided essential input to the computational study of internal motions in proteins by molecular dynamics (10, 11). Overall, the experimental data available from analysis of protein temperature factors are in reasonable agreement with these theoretical calculations (12), which must still involve a number of simplifying assumptions and model very short time spans, in the pico-second range.

Owing to the current limitations of theoretical calculations, the empirically determined information about the relative mobility of different atoms or sites in proteins that is available from refined macromolecular structures is extremely useful for understanding the dynamic aspects of protein

structure. This information on mobility is termed the atomic temperature factor and can be given by the equation $B = 8\pi^2\overline{u^2}$, where the mean square displacement $\overline{u^2}$ is averaged over time and the crystal lattice (13). Atomic temperature factors can provide important data about the lower-frequency, concerted motions of groups of atoms, indicating the relative conformational variability of different regions (12, 14). High temperature factors indicate shallow potential wells (low energy barriers between different conformations) allowing access to multiple conformations at biological temperatures. Of course, these individual atomic temperature factors do not represent local differences in temperature within the molecule but are named for the temperature-dependence of the velocity of atomic motion and of the corresponding thermal energy available for surmounting these conformational potential energy barriers. For simplicity in discussing experiments using peptides corresponding to regions of a protein, we will sometimes use the terminology *hot* and *cold* to refer to highly mobile (high temperature factors) and well-ordered regions (low temperature factors), respectively.

In addition to dynamic conformational variation, temperature factors can incorporate diverse factors that affect the measured X-ray scattering intensity—static disorder in the crystal, error in the absorption corrections, improper scaling, and errors in the interpretation of the electron density. However, the differential mobility of various regions of the molecule can be isolated from these other factors by examining the overall pattern of atomic temperature factors in highly refined protein structures that have been solved independently in different crystal systems (15).

Based upon the information from single crystal diffraction methods as well as other techniques, functional roles for flexibility are becoming known for a number of systems. Thus, for tobacco mosaic virus coat protein a transition between ordered and disordered states is known where the RNA binding segment exists in a single conformation only in the presence of RNA (16). In the tomato bushy stunt virus coat protein, flexibility is implicated both in RNA binding and in viral assembly (17). The crystal structure shows two distinct states of an N-terminal peptide fragment, that is ordered in 60 subunits and disordered in 120 subunits of the intact viral capsid. The domain flexibility of IgG, which has long been known from electron microscopic and hydrodynamic evidence, has also been directly observed in crystal studies. Fab conformations in crystal structures of intact Kol, the Kol Fab fragment, and the McPC 603 Fab fragment differ in McPC "elbow" angle (between the constant and variable domains) by up to 60° (9). Numerous other data including nanosecond fluorescence spectroscopy (18) indicate that the flexibly linked immunoglobulin domains allow the binding of ligands with diverse arrangements of the antibody molecule. In fact, the intact Dob immunoglobulin, from which a crystal structure of

the whole IgG was determined, has a substantial deletion in the hinge region resulting in greatly limited flexibility. Without such a deletion, intact immunoglobulins are extremely difficult to crystallize. The two successful cases showed ordered Fab regions and disordered Fc regions that are invisible in the electron density maps (19, 20).

The domain flexibility seen in IgG is in fact a common feature of covalently linked protein domains. With a high degree of segmental flexibility, noncovalently linked domains would tend to fall apart, but such flexibility is not a problem for covalently linked domains. Flexibility between domains has been shown to be important for substrate binding, allosteric control, and the assembly of large structures (21). For example, in hexokinase the binding of glucose causes the two domains to close around the substrate and to enclose it almost completely (22). Motion within domains is also becoming increasingly well documented. The catalytic process of carboxypeptidase apparently involves motion of the active site tyrosine (23). In adenylate kinase and lactate dehydrogenase, the appropriate enzyme-substrate stereochemical complementarity is generated by large movements of surface loops (21). In hemoglobin, the binding of oxygen to the heme iron requires sufficient mobility to open a pathway that is closed in the X-ray structure (24). In general, analysis of enzyme-substrate interactions indicates that limited protein mobility can allow the substrate access to a tightly complementary active site (25–29). However, not all intermolecular interactions benefit from mobility. In superoxide dismutase, catalysis requires the enzyme's very rapid interaction with a small substrate. Consequently, the enzyme's catalytic site is highly ordered, suggesting that the reaction does not involve significant conformational changes (30).

There are now a number of refined X-ray structure coordinates with known temperature factors that represent an excellent data base for both experimental and theoretical studies of antibody-antigen interaction. Characterization of patterns of protein mobility in a data base of 20 proteins indicates that temperature factors can reliably reflect mobility in refined, high resolution X-ray structures (31, 32). The general approach was to map the temperature factors onto the molecular surface (33), which represents the portion of the molecule available for interaction with solvent and other molecules. The resulting pattern of temperature factors was examined together with the underlying stereochemical restraints from hydrogen-bonding and packing interactions. This analysis revealed that protein surfaces characteristically show regions of both high and low mobility. As expected, regions of low mobility are often, but not always, associated with interfaces between subunits in multimeric proteins or between adjacent molecules in the crystal lattice. Those molecular surface regions that are affected by crystal contacts appear to be characterized by a lower than average ratio of side-chain to main-chain temperature factors.

Apart from these crystal and subunit contact areas, the average main-chain atomic temperature factors appear to reflect the expected relative mobility of different parts of the molecular surface based upon the nature of restraints from underlying stereochemical interactions. Regions of highest mobility are usually associated with surface projections, and surface-exposed regions of highest order often lie in grooves or valleys.

Analysis of the relationship of atomic mobility to the antigenicity of sites in proteins can be pursued with a variety of approaches. This review includes (*a*) a description of direct experimental work on the protein myohemerythrin (MHr) that was designed to use the predetermined specificity of antibodies to synthesized peptides and (*b*) retrospective studies on a number of proteins which combine previously observed immunological data and the results of high-resolution crystallography. Both of these approaches have proven valuable in developing our present picture of protein mobility as a component of protein antigenicity.

MYOHEMERYTHRIN: A PROTEIN MODEL FOR ANTIGENICITY

In the selection of a protein for detailed experimental study of mobility and antigenicity (32), the following points were considered. Mammalian proteins and proteins with considerable sequence homology to mammalian proteins were excluded to reduce the possibility that the B-cell response of experimental animals (rabbits) had been made tolerent to the intact antigen. Proteases were also eliminated since they could complicate the immunoassays. MHr was selected from the remaining data base of known protein structures for the following features: well-refined temperature factors, a wide range of mobility at the molecular surface, and contiguous regions of the sequence characterized by either high or low mobility.

MHr is a member of the hemerythrin family of proteins, which function as oxygen carriers in four invertebrate phyla. The structural fold for all of these proteins is an antiparallel bundle of four α helices (34) (named A, B, C, and D in sequence order) surrounding a two-iron center at the active site, with a loop region at the N-terminus, and shorter loops between the helices and at the C-terminus. Three-dimensional crystal structures with temperature factors have been refined at high resolution for two members of the hemerythrin family: monomeric MHr from the marine worm *Themiste zostericola* (35, S. Sheriff, W. A. Hendrickson, J. L. Smith, unpublished results) and octameric hemerythrin from *Themiste dyscritum* (36). A rigorous analysis of mobility in these two high-resolution crystal structures by Sheriff & Hendrickson together with their collaborators at the University of Washington (35) allowed the determination of consistent temperature factors after correction for crystal contacts.

The differential mobility of MHr (Figure 1) is typical of the patterns seen in high-resolution protein structures: different intradomain structural elements show significant variation in their mobilities. The temperature factors, before adjustment for the effects of crystal contacts, indicate that the A and D helices are relatively highly ordered, the C helix is highly mobile, and the B helix varies from being relatively well ordered at the N-terminal end to relatively mobile at the C-terminal end. Regions involving residues 1–17 (N-terminal loop), 38–50 (N-terminus of the B helix), and 85–94 (C-D loop) show the greatest increase in relative mobility when corrected for crystal contacts. Examination of the exposed molecular surface and corrected average main-chain temperature factors by residue indicates that a large portion (over 80%) of the molecular surface is relatively mobile. This corrected pattern of mobility is consistent with a qualitative analysis of the local and underlying stereochemical interactions.

Selection and Synthesis of Myohemerythrin Peptides

Peptide selection in the MHr system (32) was based on the main-chain temperature factors averaged by residue, since the main-chain mobility reflects the global conformational variability of the protein. Both helical and nonhelical peptide regions were synthesized. Three of the hot peptides (representing a region of high mobility in the protein) were chosen to include residues liganding the two irons; in the native protein the mobility of these sequences is limited to conformations that do not disrupt the metal ligand geometry.

The characteristics of the peptides selected for study are shown in Table 1. Highly exposed amino acid residues tend to be less hydrophobic (more hydrophilic) and—due to their external, relatively unconstrained position—more highly mobile than internal residues (40, 41). Peptides were specifically chosen to minimize differences in average exposed area and hydrophobicity per residue. Although there is some correlation of exposed area per residue and mobility among the selected peptides, this is much stronger for side-chain than for main-chain mobility. In fact, peptides with very similar average exposed areas may either be cold (peptides 22–35, 26–35, and 96–109) or hot (7–16, 42–51, and 57–66). As shown in Table 1, there is very little correlation between temperature factor and average hydrophobicity per residue for the peptides synthesized for antigenicity studies.

Regions of High Mobility Are Most Reactive with Antipeptide Antibodies

The antipeptide antisera raised against the MHr peptides were originally assayed for reactivity against the homologous immunizing peptide by enzyme-linked immunoabsorbant assay (ELISA). All but one of the synthetic peptides were immunogenic (Table 2); their relative immuno-

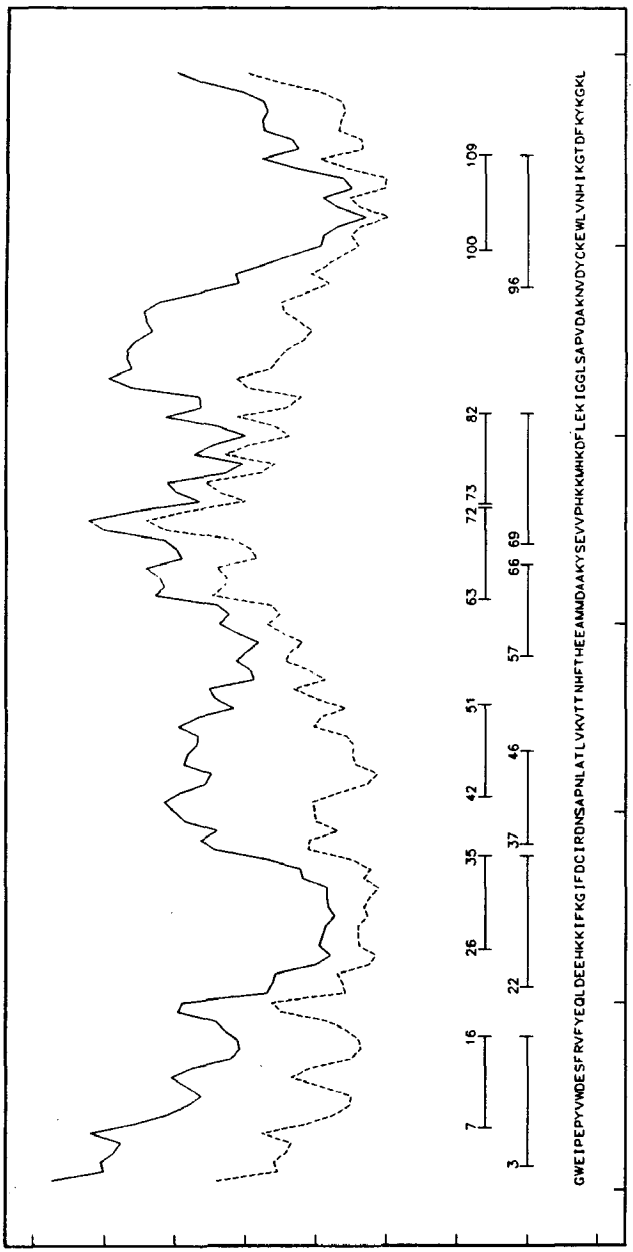

Figure 1 A plot adapted from Sheriff et al (35) of the average main-chain temperature factors along the polypeptide chain of MHr before (dashed line) and after (solid line) adjustment for crystal contacts. Regions 1–17 (N-terminal loop), 38–50 (N-terminus of the B helix), and 85–94 (C-D loop) show the greatest increase in relative mobility when corrected for crystal contacts. The 118-residue amino acid sequence of MHr (37), as corrected by a sequence inversion of residues 34 and 35 revealed by X-ray crystallographic studies (J. L. Smith & W. A. Hendrickson, unpublished results), is shown in one letter code. The 10 and 14 residue sequences of the protein chosen for peptide synthesis are indicated by sequence-numbered bars.

Table 1 Myohemerythrin peptide data[a]

Sequence Position	Secondary Structure	Mobility				Average Exposed Area	Average Hydrophobicity
		Type	Main*	Main	Side*		
3–16	N-term	Hot*	30.4	19.9	36.2	52.3	0.02
7–16	N-term	Hot*	28.6	18.8	32.4	43.8	0.02
22–35	A helix	Cold	20.4	16.8	26.2	40.6	−0.16
26–35	A helix	Cold	20.1	16.6	26.2	43.8	0.05
37–46	A loop	Hot*	28.9	18.5	30.7	59.7	−0.23
42–51	B helix	Hot*	28.4	17.8	31.8	44.9	0.26
57–66	B helix	Hot	28.0	24.2	30.2	45.6	−0.11
63–72	B-C loop	Hot	31.9	27.4	34.1	60.0	0.05
69–82	C helix	Hot	29.6	26.2	35.9	54.9	−0.18
73–82	C helix	Hot	27.9	24.9	35.2	55.4	−0.41
96–109	D helix	Cold	20.8	17.6	26.7	40.1	0.04
100–109	D helix	Cold	19.4	16.9	25.1	36.7	−0.01

[a] Peptides are named by their respective sequence position in MHr. All mobilities are derived from the temperature factor data by Sheriff et al (35). Peptides are classified into three types of mobility: hot (high average main-chain temperature factors), cold (low average main-chain temperature factors, and hot/contact, abbreviated hot* (high average main-chain temperature factors after correction for the effects of crystal contacts). Mobility in $Å^2$ is expressed as average main-chain* (calculated from the atomic temperature factors for N, Ca, C, O atoms adjusted for crystal contacts); average main-chain (calculated from the atomic temperature factors for N, Ca, C, O atoms); and average side-chain* (calculated from the atomic temperature factors for side-chain atoms adjusted for crystal contacts). Average exposed area in $Å^2$ is calculated from the molecular surface program MS (38). Average hydrophobicity is calculated using the Eisenberg consensus scale (39). Properties were first averaged for each residue, then these residue averages were averaged for each peptide.

genicity did not correlate with the mobility of the corresponding MHr residues. All antisera raised against cold peptides (22–35, 26–35, 96–109, and 100–109) gave negative or low reactivity with the protein, and all antisera raised against hot peptides (excluding peptide 42–51, as not immunogenic) gave higher reactivity against the protein. Of the antisera against hot peptides, those with the lowest reactivity against MHr (57–66 and 63–72) also have the lowest reactivity against their respective homologous immunizing peptides. In addition, the avidity of the anti-peptide antisera against the protein matched the pattern of relative reactivity; antisera with higher titers generally showed greater avidity (32).

The antigenic reactivity of the anti-peptide antibodies with the native protein was also tested in solution under conditions that did not affect the iron center. The reactivity and specificity seen in these immunoprecipitation studies and the associated gel electrophoresis analysis (Table 2) were consistent with the ELISA analyses. Antisera against hot peptides react more strongly with MHr, than do antisera against cold peptides. The striking correlation seen in both types of immunoassay between the

Table 2 Reactivity of MHr anti-peptide antisera

	ELISA titers[a]		Immunoprecipitation	
Sequence position	α Peptide vs peptide	α Peptide vs MHr	^{125}I-MHr precipitated cpm[b]	% Inhibition by peptide[c]
Hot/contact peptides				
3–16	640–1280	12800	14849	97.1
7–16	640–1280	3200	7384	92.6
37–46	640–1280	9500	4026	100.0
42–51	very low[d]	very low	0	0.0
Hot peptides				
57–66	160–320	600	7277	80.0
63–72	160–320	375	2543	77.6
69–82	>2560	1050	3774	91.3
73–82	>2560	1400	2154	62.0
Cold peptides				
22–35	1280–2560	very low	180	0.0
26–35	320–640	60	0	0.0
96–109	1280–2560	200	2617	22.5
100–109	320–640	very low	0	0.0

Data from Tainer et al (32).

[a] ELISA titers are expressed as the reciprocal of antibody dilution extrapolated to bind 50% of 50 pmole antigen per well.

[b] Average of two independent experiments, after correction for nonspecific binding.

[c] 100.0% minus percent of activity remaining in the presence of the peptide.

[d] Very low indicates indistinguishable from zero in our assay conditions.

mobility of sites in MHr and their reactivity with anti-peptide antibodies is shown in Figure 2. The reactivities of the anti-peptides antibodies are mapped onto the alpha carbon backbone and the average adjusted main-chain temperature factors are mapped onto the external molecular surface. Of the cold peptides, only antibodies to peptide 96–109 have significant reactivity with intact MHr; the N-terminal end of this peptide has four residues with higher temperature factors. Of the hot peptides, the N-terminal loop peptide 3–16 represents the target site with both the highest mobility and the highest reactivity with anti-peptide antibodies. Reduced mobility due to structural constraints in MHr resulting from the metal liganding may modulate the degree of antigenic reactivity. In the immunoprecipitation assays, antisera against the shorter of the two nested peptides containing two metal ligands in the intact MHr (73–82) have the lowest anti-protein reactivity of any hot peptide antisera. All of these results appear to support the correlation between the mobility of sites in a protein and their reactivity with anti-peptide antibodies.

MAPPING PROTEIN MOBILITY AND ANTIGENICITY

Existing data are not sufficient to analyze many systems rigorously for the correlation of structural mobility and antigenicity. However, as shown below, most of the information available is consistent with this correlation. In practice, the probable mobility of certain areas of a protein structure can often be estimated from the local and underlying stereochemical interactions, shape, and surface exposure, if an accurate atomic structure is available. Analysis of the crystallographic temperature factors in a number of different proteins indicates that flexible areas can often be correctly identified in this manner (31, 32). In addition, the N- and C-termini of proteins are almost uniformly capable intrinsically of high mobility, since they are the only sections of the polypeptide chain that in general lack covalent attachment. Moreover, comparative studies show that these termini are often located at the surface and show a relatively high flexibility (45).

Many antigenic determinants involve these mobile N- and C-terminal residues (31). The N-terminal regions of both bovine pancreatic ribonuclease (46) and mammalian cytochrome *c* (47) bind antibodies raised in rabbits against the intact molecule. Similarly, antigenic determinants involving active site regions known to be mobile are common. Examples include monoclonal antibodies that bind in the active site region of neuraminidase (48), carboxypeptidase A (49), hexokinase (50), as well as the NADPH-binding region of dihydrofolate reductase and glucose-6-phosphate dehydrogenase (51). In the case of carboxypeptidase A, monitoring the CD spectrum of an arsenilic derivative of the catalytically essential Tyr 248 allowed the identification of a conformational change at the enzyme's active site resulting from the binding of either of two different monoclonal antibodies (49).

Since both mobility and antigenicity are properties associated primarily with the structure rather than with the linear sequence of a protein, their correlation is best analyzed using three-dimensional modeling on computer graphics. Based upon the data from contiguous determinants, it has been suggested that an antigenic determinant involves about six amino acid residues (52). Logically, the influence of site mobility on antigenic interaction need not depend solely on the mobility at a given residue, but may include movements of residues adjacent in sequence as well as of spatial neighbors resulting from the three-dimensional fold. The mobility of neighboring residues may act to increase the exposure of an epitope or to improve the general complementarity of the contact region between antigen and antibody. The current problems with any correlation of

structure and antigenicity involve limitations in the available data: the inability to define accurately the mobility of sites involved in crystal contacts, the imprecise definition of antigenic sites in some instances, and the general statistical limitations of data obtained using monoclonal antibodies.

Although the antigenicity of proteins is influenced by evolutionary variance (53), this factor may be related to site mobility. Owing to the phenomenon of immunological tolerance, antibodies may not be elicited against those regions of the antigen that share structural or chemical properties with self-molecules of the host (54, 55). Thus, for proteins that are homologous to host proteins (e.g. cytochrome *c*, myoglobin, lysozyme, and insulin), tolerance may strongly direct the immune response against sequence-variable regions. However, polymorphism in evolutionarily variant proteins is related to polypeptide chain flexibility, since natural selection favors those mutations that do not perturb the overall conformational stability of a protein or reduce its functional capacity through structural changes. Indeed, amino acid sequence variability is most likely to persist in those regions of the molecule where local changes in conformation can be tolerated. In addition, such changes are most likely to occur on the surface regions of the molecule and to be accessible with the B-cell immunoglobulin receptor.

Other factors that appear to influence antigenicity also correlate with mobility. Hydrophilicity (or hydrophobicity) indices (6, 56, 57, 58) have been in wide use in recent years for predicting the exposed surface regions of proteins and consequently the potential antigenic sites. Regions of the polypeptide chain that are highly exposed often have few interactions with the rest of the protein and are therefore relatively mobile. Thus, while surface topography is correlated with antigenicity (E. D. Getzoff, J. A. Tainer, A. J. Olson, unpublished results), this relationship may largely reflect the high mobility of projecting regions.

In the following paragraphs, we summarize the patterns of antigenicity and mobility of 12 known three-dimensional protein structures for which antigenic determinants are also known, using computer graphics to illustrate examples of the correlation between mobility and antigenicity in these proteins.

Insulin

Insulin is a peptide hormone with a fold dominated by disulfide bonds (21), two of which link the separate A (21 residues) and B (30 residues) polypeptide chains. The well-defined, 1.5 Å resolution structure (59, 60) of porcine insulin reveals a hydrophobic core and two predominantly hydrophobic surfaces that are involved in dimeric and hexameric contacts

and may be important in the binding of the active monomeric hormones to receptor (61). Most hydrophilic residues are on the surface exposed by the hexameric form of the molecule. The sequences of more than 28 species have been determined (62) and indicate that the disulfide bridges and hydrophobic core of the monomer are invariant. The highly variable residues for most mammalian insulins are residues 8–10 on the A chain and residues 29–30 on the B chain. These evolutionarily variable residues are thought to have little effect on the ability of the monomer to bind to its receptor (61), but have been shown to be important in the antigenicity of the molecule.

Using a panel of 18 monoclonal antibodies raised in mice to bovine and human insulin, Schroer and her coworkers implicate epitopes involving residues A4, the A-chain loop (residues A8–A10), B3, and residues B28–B30 (63, 64). Other experiments with insulin derivatives shortened at the N-terminal end indicate the importance of B1 in antigenic recognition (65). As shown in Figures 3, 4, all of these antigenic determinants occur in local temperature factor maxima along the sequence, and all fall along a strip of the molecular surface showing high mobility (31). In their structural analysis of insulin determinants, Talmon and her colleagues (68) noted the conformational disorder of the insulin determinant involving residues B29 and B30. Plots of hydrophilicity and exposed area (Figure 3) indicate that antigenicity correlates better with mobility than with either of these other parameters.

Cytochrome c

Cytochrome *c* (M_r 12,800) is a compactly folded polypeptide of 104 residues (in mammalian species) containing a heme group that is essential to the structural integrity of the molecule. The sequences of more than 75 eukaryotic cytochromes are known (62, 69) and the crystal structures of one mammalian (70) and two piscean species (70, 71, 72) have shown essentially identical folding except for expected changes in side-chains where sequence differences occur. In fine-specificity immunological studies with evolutionarily variant cytochromes, Margoliash and others (73, 74, 75, 76, 77, 78b) have identified three important sites of antigenicity. The first includes residues 89 and 92 and occurs at the N-terminal end of the C-terminal α helix; the second lies around residues 60 and 62 at the N-terminal end of a distorted helix; and the third involves residue 44, which forms part of a beta bend. The third of these regions has been successfully modeled by synthetic peptide sequences (79, 80). The synthetic pentapeptide that represents 42–46 in the rat protein is the shortest sequence containing residue 44 that binds anti-rat cytochrome *c* antibodies raised in rabbits (80). Two of the three immunodominant regions, residues 44 and 89/92, lie in areas of the

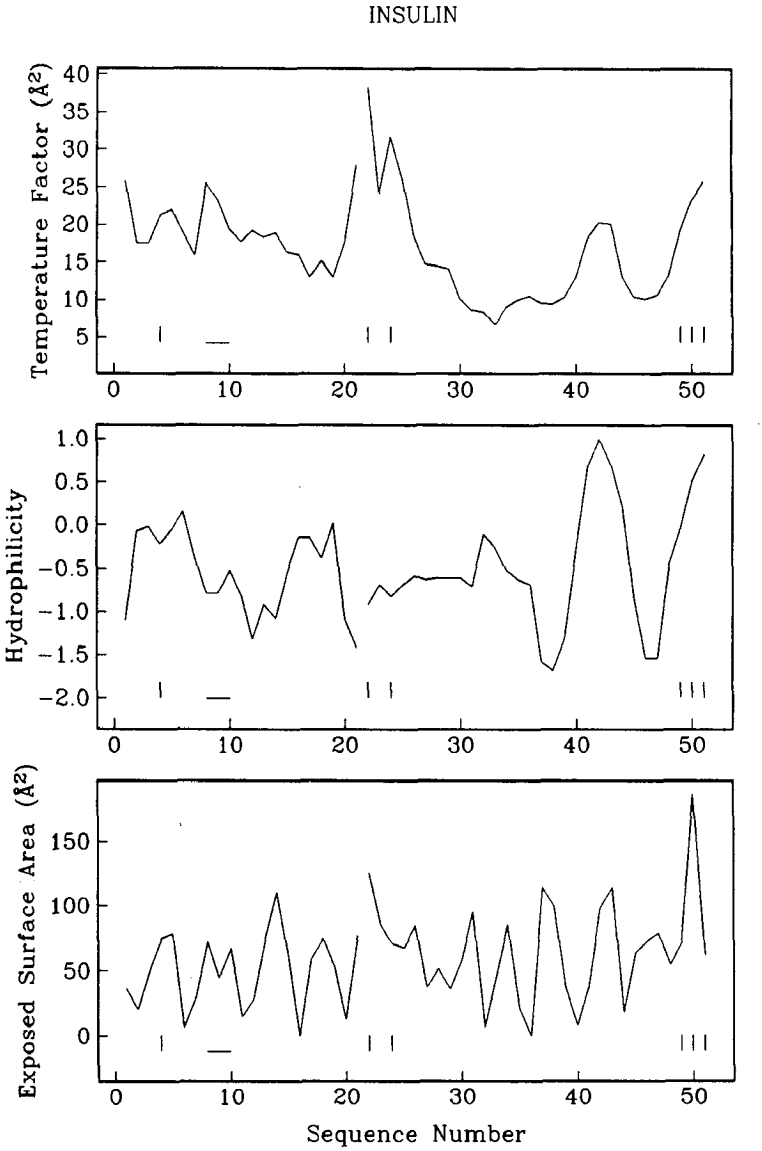

Figure 3 Plots of the linear relationship of temperature factor, hydrophilicity and exposed area to sequence position in the insulin A and B chains. A chain residues are numbered 1–21, B chain residues are numbered 22–51. Contiguous determinants are shown as horizontal bars: discontiguous (conformational) determinants as vertical bars. Coordinates are taken from (66) hydrophobicity values from (39), and algorithm for exposed area calculations from (38).

polypeptide backbone where high mobility occurs as measured by average main-chain temperature factors (Figures 5 and 6). The third immunodominant region, residues 60/62, is involved in a crystal-packing contact and shows high mobility in molecular dynamics calculations (81), and in NMR experiments (82). Indeed, an early suggestion of the possible importance of protein flexibility in antigenicity came from these NMR studies by Moore & Williams (82).

In a study of rabbit responses to cytochrome *c* as a self-antigen, Jemmerson and Margoliash (78) showed that self-antigenic sites on rabbit cytochrome *c* correspond to areas of sequence variation in mammalian cytochromes. These data suggest that only certain regions of a self-antigen elicit immune responsiveness, i.e. those that allow sequence variation and are therefore likely to have fewer structural restrictions on their conformation.

Recently, both the N- and C-termini have also been identified as antigenic determinants. Most of the antibodies raised in rabbits against pigeon cytochrome *c* were shown by fine-specificity studies with intact proteins to be directed against a determinant composed of the residues 3, 103, and 104 (78a). The evolutionarily conserved N-acetylated amino-terminus has been shown to be both antigenic and immunogenic (47). Because of the lack of suitable probes this region of the molecule in mammals had not been observed to be antigenic. In studies with synthetic peptide antigens, from 5–95% of the total antibody populations in different antisera were found to bind to residues 1–4 of cytochrome *c*. The variability among antisera was attributed to different forms of the injected protein, since cytochrome *c* was used polymerized with glutaraldehyde for effective immunization. As previously discussed, these determinants on both the N- and C-termini are likely to be highly flexible due to their exposed location and lack of covalent attachment. The antigenic determinant on the C-terminus involves the most highly mobile residue (Figures 5, 6); the apparently low mobility of the N-terminus shown in Figure 5 may result from the crystal contact there.

Myoglobin, Hemoglobin, and Leghemoglobin

Myoglobin is an α helical protein of 153 amino acid residues that is structurally related to a single hemoglobin subunit. Three-dimensional structures of myoglobin are known for both the sperm whale (83) and seal (84). Crumpton & Wilkinson (85) first identified two major antigenic determinants of myoglobin using sperm whale as the source of the protein. These sites were confirmed by Atassi (52), who proposed that the entire antigenicity of myoglobin resided in the regions comprised of residues 15–22, 56–62, 94–99, 113–119, and 145–151; all of these are short contiguous

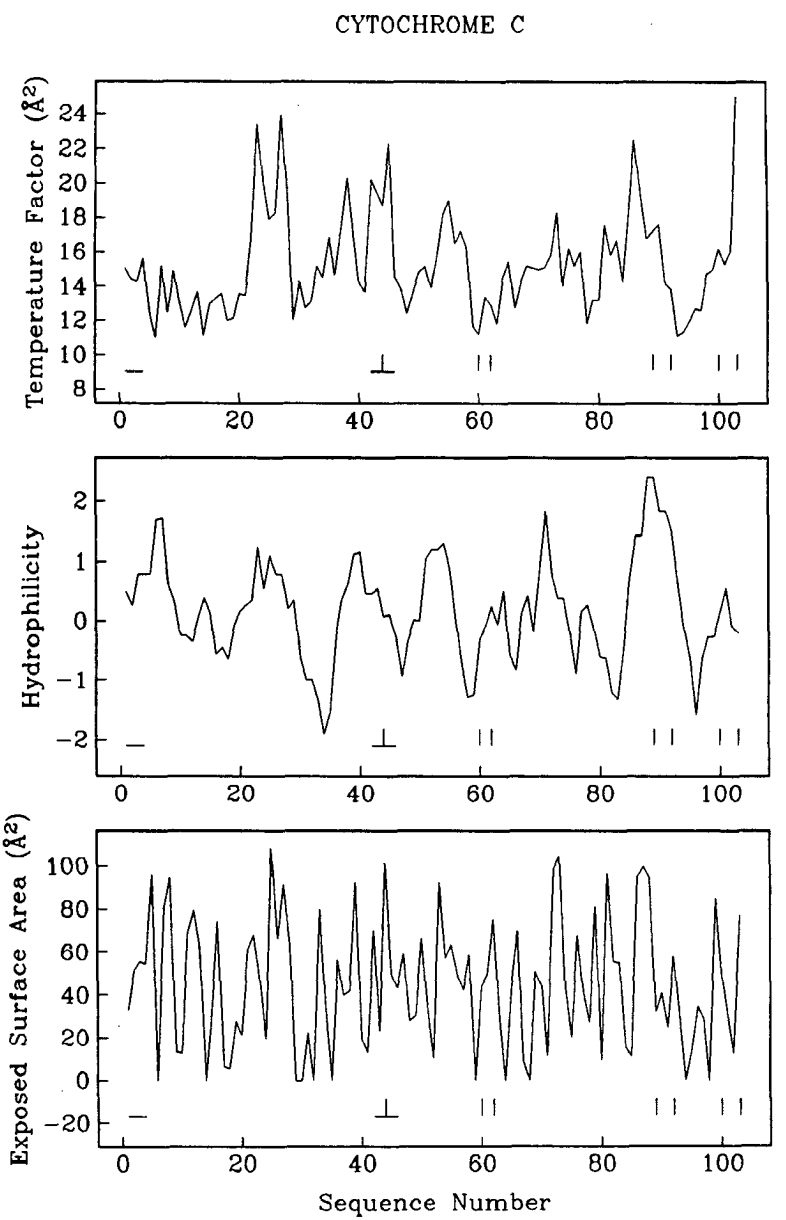

Figure 5 Plots of temperature factor, hydrophilicity, and exposed area versus sequence position in cytochrome *c* (see legend for Figure 3).

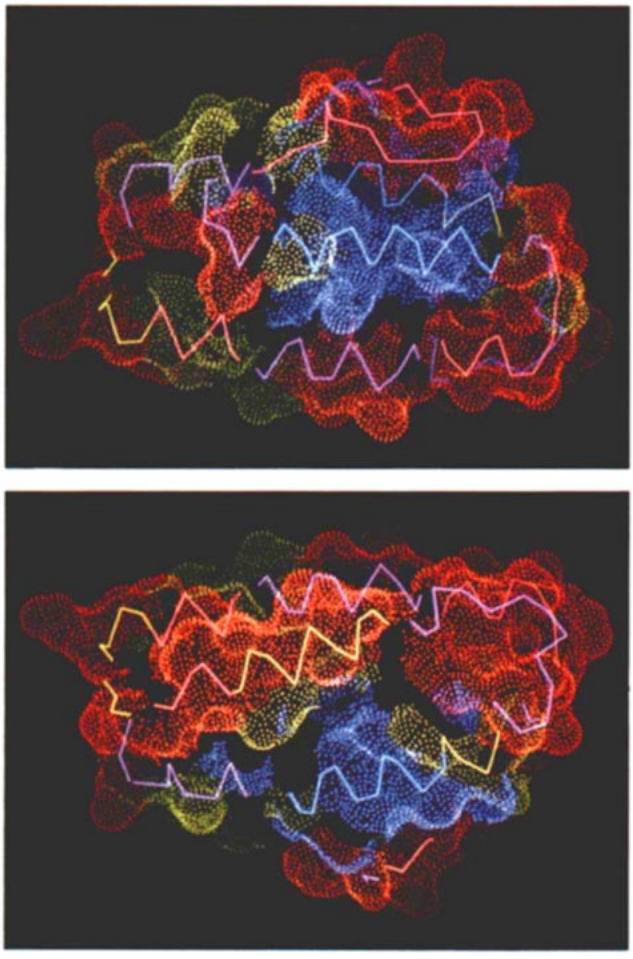

Figure 2 Correlation between antigenicity and mobility in MHr (32). The protein skeletal model (colored lines) and molecular surface (38,42) (colored dots) were displayed using the computer graphics language GRAMPS (43) and the molecular modeling program GRANNY (44). The alpha carbon backbone is color-coded by antigenicity based upon immunoprecipitation results (see Table 2): red = high (>3,500 c.p.m.), yellow = medium (2,000 to 3,000 c.p.m.), and blue = low (<200 c.p.m.), magenta = not studied. The exposed molecular surface is color-coded by average adjusted main-chain temperature factors: red = hot ($\geq$27 Å^2), yellow = medium (22 up to 27 Å^2) and blue = cold (<22 Å^2). The MHr molecule has been dissected into individual peptides (backbone and molecular surface) and slightly exploded to illustrate the correlation of temperature factors with antigenicity. Top view, the N-terminus (red backbone and surface, upper right), the A helix (blue backbone and surface, center), the A loop (red backbone and surface, lower right), and the C-terminal end of the B helix (red and yellow backbone and surface, lower left). Bottom view (opposite face), the non-overlapping fragment of the B-C loop (yellow backbone and red surface, left), the C helix (red and yellow backbone and surface, middle), and the D helix (blue and yellow backbone and surface, lower right).

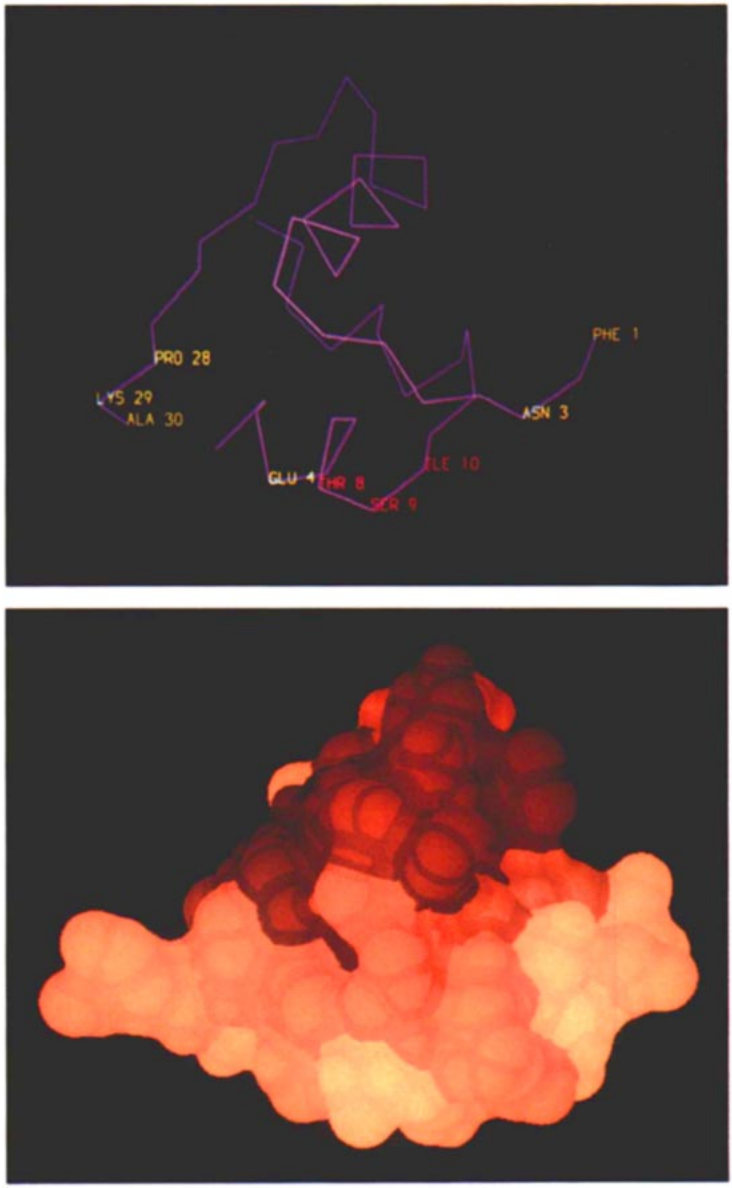

Figure 4 Insulin alpha carbon backbone (top view) and solid external molecular surface (bottom view) in the same orientation. Residues are labeled (top view) in red for contiguous and in yellow for discontiguous determinants. The surface is color-coded by the average main-chain temperature factors using a radiating body color scale. The highly mobile regions (lightest color) are associated with the antigenic determinants. Vector graphics views (top and in Figures 6 and 8) used the programs GRAMPS (43) and GRANNY (44). Raster views of the solid molecular surface (bottom and in Figure 6) used Connolly's programs AMS (42) and RAMS (67).

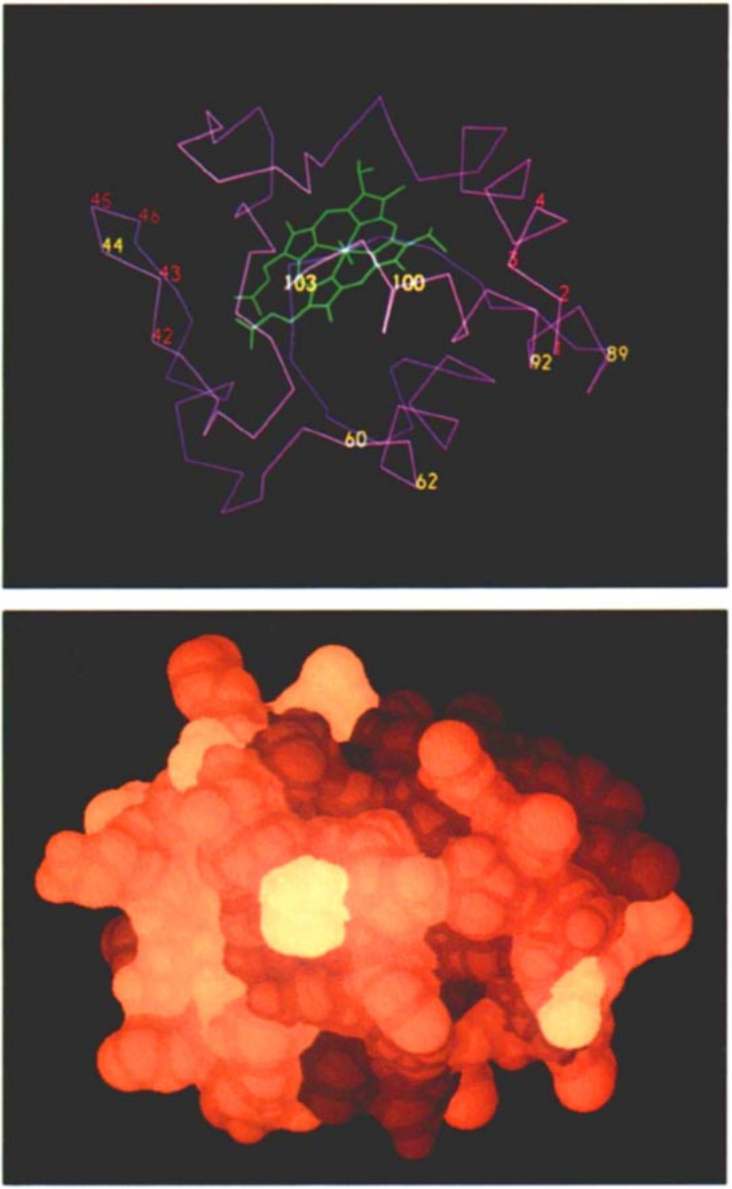

Figure 6 Cytochrome *c* alpha carbon backbone (top view) and solid external molecular surface (bottom view) labeled and color-coded as in Figure 4. The bonds of the heme are shown in green. The highly antigenic site at residue position 103 (center) is the most mobile.

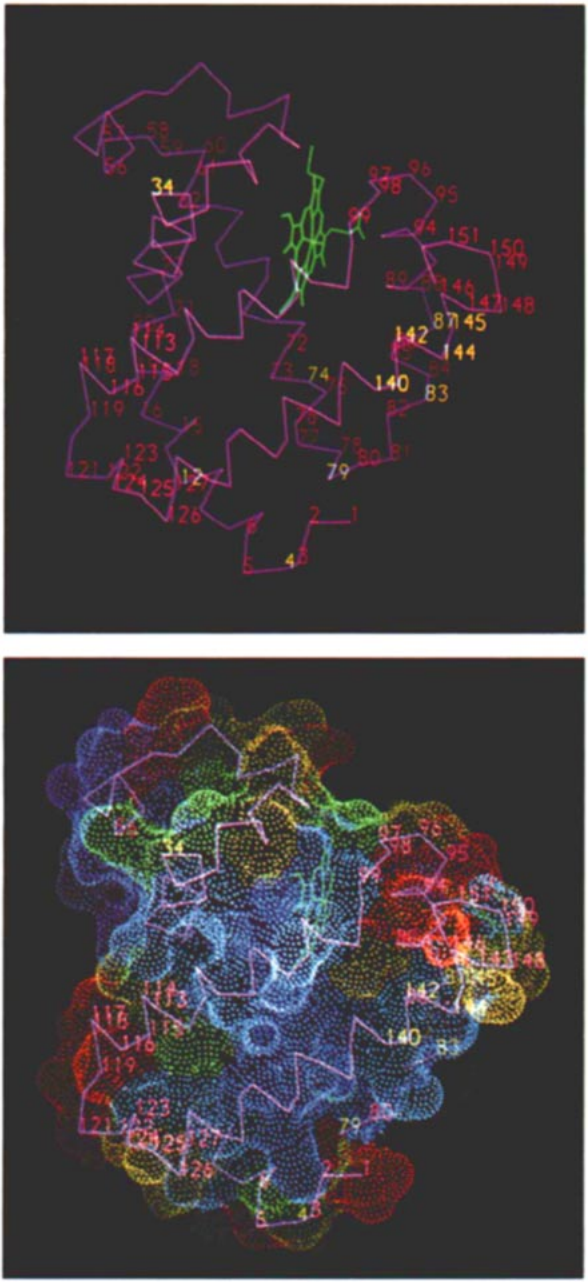

Figure 8 Myoglobin antigenic determinants cluster at highly flexible regions. The heme bonds (green) and alpha carbon backbone (magenta) are shown with residues labeled pink for contiguous and yellow for discontiguous determinants. The molecular surface (bottom view) is shown as dots color-coded by average main-chain temperature factors (highest to lowest in order red, yellow, green, cyan, blue).

sequences on the surface of the molecule. Later work by Atassi and coworkers (86) showed that antibodies raised to surface peptides 1–6 and 121–127 also cross-reacted with native myoglobin even though these peptides did not react with antimyoglobin antibodies.

Using mammalian myoglobins that have closer sequence homology with the host protein than does sperm whale myoglobin, Leach and coworkers have demonstrated that other residues, outside these five contiguous regions, are involved in binding to anti-bovine myoglobin antibodies (87). In addition, mouse monoclonal antibodies to sperm whale myoglobin did not bind to any cyanogen bromide–cleaved contiguous fragments of the molecule. Fine-specificity studies with these monoclonal antibodies indicated discontiguous antigenic sites that involve residues at positions at 4, 12, and 79; at 83, 144, and 145, and at 140 (88, 89). Monoclonal antibodies to human myoglobin recognized two sites: one that includes residues 34 and either 113 or 53, and one in which 74, 87, and 142 are involved (90). Only two of the residues (residues 113 and 145) implicated in the binding of these two sets of monoclonal antibodies to human and sperm whale myoglobin are within the sites defined by Atassi. A synthetic peptide representing residues 72–89 of bovine myoglobin binds to rabbit anti-bovine myoglobin antibodies (91).

It has been suggested (3) that almost all the exposed molecular surface of myoglobin may be antigenic given suitable differences between the host and immunizing protein. However, as shown in Figure 7, high temperature factors are found at all but one of the contiguous peptide sequences originally shown to be antigenic, i.e. four of the five sites proposed by Atassi, the region between 72–89 (91), and the two sites between 1–6 and 121–127 (86). The remaining contiguous determinant (residues 56–62) includes a crystal contact of this corner between the D and E helices. The correlation of antigenicity with mobility for the six sites can be seen in Figures 7, 8; in general the antigenicity of these sites also correlated with the related parameters of hydrophilicity and to a lesser extent of exposed surface area. Of the discontiguous or conformational sites, all involve relatively mobile residues except for the site at residue 140, which is involved in a crystal packing contact.

Based on the structural homology between myoglobin and single subunits of hemoglobin, regions in the alpha and beta chains of human hemoglobin equivalent to myoglobin site 15–23 were predicted to be antigenic sites (92). Later studies concluded that the sites in hemoglobin analogous to all five. Atassi antigenic sites in myoglobin are also antigenic (93). These sites in hemoglobin show the same general pattern of relatively high mobility seen in myoglobin.

Leghemoglobin is an oxygen binding, monomeric plant protein that is

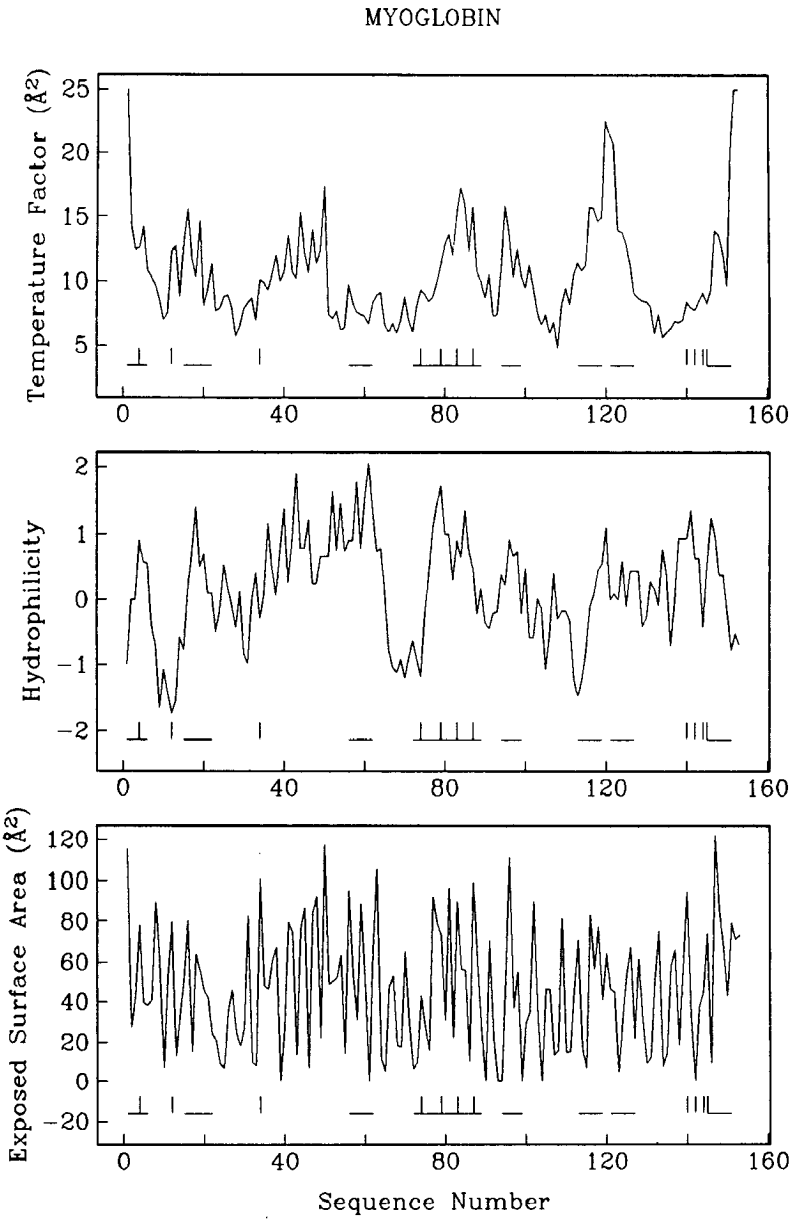

Figure 7 Plots of temperature factor, hydrophilicity, and exposed area versus sequence position for myoglobin (see legend for Figure 3).

found in the root nodules of leguminous plants. Several refined 2 Å resolution structures have been determined for this protein (94). Since leghemoglobin shows sequence homology and similar overall polypeptide chain conformation to myoglobin (95), the antigenic sites identified for myoglobin may also pertain to this related molecule (96). Five peptides corresponding to the following regions in the polypeptide chain (15–23, 52–59, 92–98, 107–116, and 132–143) were synthesized and shown to be reactive with anti-soybean leghemoglobin antibodies. Three of these sites (15–23, 52–59, and 92–98) are associated with temperature factor maxima. The remaining two sites (residues 107–116 and 132–143) are involved in crystal contacts.

Lysozyme

Lysozyme (M_r = 14,300) is an antiparallel α helical protein of 129 residues internally cross-linked by four disulfide bonds between residues 6–127, 30–115, 64–80, and 76–94. The primary sequence is known for ten avian lysozymes (97), and crystallographic studies of human (98, 99), turkey (100, 101), chicken (102, 103), and bacteriophage lysozyme (102) show similar tertiary folding in the molecule. Although the highest resolution lysozyme coordinates and temperature factors have not been distributed through the Brookhaven Protein Data Bank, sufficient published information exists to define the local mobility of sites. Our analysis is therefore based upon the Protein Data Bank structure of Rao et al (104), which does have temperature factors, as well as on the published plots of Artymiuk and colleagues (15), and the dynamics calculations of the Karplus group (105).

In one of the now classic studies of protein mobility, the Oxford X-ray group examined the atomic displacement in the crystals of hen and human lysozyme (15). They found that the temperature factors gave valid indications of molecular motion, and that this mobility was associated with the lips of the active site formed by those main-chain segments that undergo conformational change when ligands are bound. They also noted that the region of highest mobility represents the principle antigenic determinant of lysozyme identified by Atassi & Lee (5).

The antigenicity of lysozyme has been exhaustively studied. The first region of the molecule shown to be antigenic and immunogenic is the so-called loop region from residue 64–82, which is stabilized by the 64–80 sulfhydryl link (106, 107, 108, 109, 110, 111) and binds from 5–14% of the antibodies in a typical antilysozyme serum (112). Three sites on the lysozyme surface have been proposed (113) as the sole locations of antigenicity of the molecule. They comprise residues at the following positions: 5, 7, 13, 14, and 125 in Site I; 33, 34, 113, 114, and 116 in Site II;

and 62, 87, 89, 93, 96, and 97 in Site III. These findings have been challenged by other workers (97, 112, 114, 115) who have clearly identified as antigenically important other residues on the surface of lysozyme, which are remote from these regions; they have estimated (97) that Sites I, II, and III account for only 30–50% of the antigenic difference between pairs of lysozymes. In addition to Sites I, II and III, and the loop region, a fifth site involving the active site of the enzyme (including residues Arg 45, Asn 46, Thr 47, Asp 48, Gly 49) and Arg 68 has been identified (97, 115); 5–10% of the antibody population against lysozyme is directed against it. A minor determinant, one which binds only about 1% of hen egg white lysozyme antibodies raised in rabbits, sheep and goats (114), is the only determinant suggested for lysozyme besides the loop region, which is a contiguous sequence of the molecule; it lies between residues 38 and 54. These two contiguous determinants occur at major peaks in the main-chain temperature factors. From molecular dynamics calculations (105) the mobility of residues 96 and 97 in Site III is known to be high.

Lactate Dehydrogenase (LDH-C_4)

LDH-C_4 (lactate dehydrogenase-C_4) consists of four subunits of the 330 residue LDH-C polypeptide each of which is folded into two domains: the first, a parallel β sheet wound on both sides with α helices, and the other containing two twisted β sheets and four α helices (21). The only two amino acid sequences of LDH-C known (116) (from rat and mouse testes) show a large number (32 residues) of sequence differences.

The isozyme LDH that has been most widely studied for immunochemical properties is the sperm specific LDH-C_4, the predominant LDH isozyme in mammalian spermatozoa. Polyclonal antisera to this isozyme do not cross react with LDH-A_4 or LDH-B_4 within the same species (117), although all three isozymes presumably have similar three-dimensional structure and a common evolutionary origin (118). Antisera raised in rabbits against mouse LDH-C_4, however, cross-react with LDH-C_4 from other mammalian species (117, 119).

The antigenicity of LDH-C_4 has been studied at the sequential level using linear tryptic peptides by Goldberg and his coworkers. Of six tryptic peptides representing residues 5–16, 44–58, 61–77, 180–210, 211–220, and 282–317 in the molecule (120), two have been shown to be antigenic. A peptide isolated from a tryptic digest of the whole LDH isozyme comprising residues 152–159 reacts with rabbit anti-mouse LDH-C_4 antisera, and, coupled to BSA, induces an antibody response (in rabbits) that recognizes the intact molecule (121). Four monoclonal antibodies from hybridomas to mouse LDH-C_4 show unique specificities on the basis of

their cross-reactivities with a panel of five isozymes (122), one of which recognizes the sequential peptide 101–115.

The antigenic region 152–159 is an eight residue surface exposed loop that connects a helix and a β strand (123). The determinant contained in 101–115 also includes a flexible surface loop region of the molecule and the first eight residues of an α helix. These regions may not represent exactly contiguous determinants in the whole molecule since they were identified by using tryptic peptides and not sequential peptide synthesis. Although crystallographic temperature factors for lactate dehydrogenase are not currently available, these determinants appear to involve flexible areas of the molecule as based upon the structural interactions and the secondary structure of the residues. According to the hydrophilicity profiles of Hopp & Woods (6), neither of these regions was identifiable as an antigenic determinant (124).

Ribonuclease

Bovine pancreatic ribonuclease is a structurally well-characterized protein of 124 amino acid residues cross-linked by four disulfide bonds. The known refined crystal structures include an X-ray structure (125) and another structure that was recently refined using both X-ray and neutron data (126).

Cyanogen-bromide fragmentation of the molecule yields four peptides: 1–13, 14–30, 31–79 and 80–124. These peptides have been used to study the conformational mobility (127) and the location of antigenic determinants in the molecule (46). Antisera to bovine ribonuclease were fractionated by immunoabsorption on these fragments covalently coupled to Sepharose. Binding data for these antibody populations with tryptic fragments of ribonuclease indicate that region 31–79 probably contains two determinants 40–61 and 63–75 and that the antigenicity of fragment 80–124 is limited to residues 80–104 (46). Using the X-ray crystallographic structure (128, 129) and derived static-accessibility data (130), Chavez & Scheraga (46) eliminated residues from these four antigenic regions that have no accessibility to solvent. This procedure, together with other structural and immunochemical considerations, indicates that within these regions the sites most likely to be antigenic are residues 1–10, which include an α helix at residues 4–10; residues 63–75, which contain interdigitated β bends at 66–68; and residues 98–104, which contain portions of a β-pleated sheet. All three of these determinants are associated with areas identified as mobile in the crystallographic temperature factors. In addition, there may be a determinant within segment 40–61 centered around residue 49 (46). The temperature factors associated with this determinant indicate high order in the crystal, but the low ratio of side-chain to main-chain temperature

factors may indicate a crystal packing contact, which we confirmed by examination of the crystal lattice on computer graphics.

Non-Mammalian Proteins

The non-mammalian origin of proteins such as the neurotoxins, ferredoxin, and tobacco mosaic virus coat reduces the possibility of immunological tolerance and thus simplifies the interpretation of their antigenic data.

Scorpion neurotoxins [crystal structure 1.8 Å resolution (131)] are small proteins of 60–65 residues cross-linked by four disulfide bridges; they have been divided into two groups, α and β, on the basis of their binding sites in the mammalian nervous system. Two antigenic sites in α toxin of the North African scorpion have been located in the region between residues 50–59 and around the disulfide bridge between cysteines 12 and 63 (132). Residues 50–59 do not show high crystallographic temperature factors; however, the more precisely determined site [involving residues 10 and 64 in one determinant (133)] is associated with the second and third highest peaks in the average main-chain temperature factors.

Snake venom neurotoxins [crystal structure at 2.2 Å resolution (134)] contain 60–74 amino acid residues and exhibit a wide degree of variance in amino acid sequence between species, although each contains four invariant disulfide bridges (135). The common disulfide pairing indicates that the three-dimensional structures of all the snake toxins are similar, but immunological evidence indicates that the "short" neurotoxins of 60–62 residues are distinct from the "long" neurotoxins of 71–74 residues, which also have an additional disulfide bridge. There is, however, good antigenic cross-reactivity amongst members of each group (135). Residues involved in one antigenic determinant have been identified as residue 16, which is associated with a plateau of high mobility, and the N-terminal amino group (136), which is associated with a peak in conformational mobility.

Ferredoxin is a small bacterial protein containing 55 amino acid residues; it is immunogenic in rabbits, guinea pigs, and mice (137). High-resolution refined crystal structures are known for *Azobacter vinelandii* (138), *Peptococcus aerogenes* (139), *Clostridium pasteurianum* (140). In a series of publications (141–143) Levy and her coworkers have established that this molecule has only two antigenic regions—the N-terminal heptapeptide and the carboxy terminal pentapeptide. This well-ordered protein consists of two α helices packed against four β strands; the two termini are probably the most highly mobile regions.

While we were completing this review, an independent study on the protein antigenicity of tobacco mosaic virus (145) was published that also identified the relationship between mobility and antigenicity. Besides analyzing lysozyme and myoglobin with conclusions similar to those given

above, these investigators examined the mobility and antigenicity of tobacco mosaic virus coat protein (TMV). The seven contiguous epitopes were examined: 1–10, 34–39, 55–61, 62–68, 80–90, 105–112, and 153–158. These epitopes correlate well with local maxima of average main-chain temperature factors and to a lesser extent with hydrophilicity.

DISCUSSION

As a result of the growth of detailed information on both antibody and antigen structure, discussions of antigenic recognition will be increasingly formulated in terms of detailed atomic geometry. In a number of laboratories, structural studies are currently in progress on monoclonal antibodies and Fab fragments raised against sites on known protein structures, and a high resolution structure of a lysozyme-Fab complex may soon be available (146, 147, 167). In addition improvements in peptide synthesis and immunoassay now allow probing for antigens at the resolution of single amino acids (148). The combination of this detailed structural and antigenic information may well produce some surprises about the structural basis for antigenic recognition.

Currently it is worthwhile to consider protein folding as a paradigm for molecular interactions. Folding is known to be determined both by the sequential order of amino acids in the polypeptide chain and by the chemical environment. In spite of this knowledge, we remain a long way from predicting a protein fold from its primary structure. We are perhaps a similar distance from a complete understanding of antigenic recognition. Presumably, the interaction of epitope and paratope changes the environmental chemistry from that of the independent solvated molecules. By analogy to protein folding, this change in environment may be accompanied by changes in local structure. The correlation of site mobility with antigenicity in proteins is consistent with the view that such macromolecular interactions involve some degree of induced fit to maximize complementarity. However, a common belief has been that protein epitopes are rigid and peptide epitopes extremely flexible. Realization that this distinction must now be abandoned takes us one more step towards understanding protein antigenicity.

Some have viewed as an apparent contradiction the finding that most monoclonal antibodies raised against the intact protein are conformation specific (3), whereas the majority of monoclonals against a representative peptide recognize the intact protein (8). In regard to the first point, it is not unreasonable to expect that most antibodies will be directed against discontiguous conformational epitopes, which are after all more numerous on the protein surface. In regard to the second point, the results with MHr

(32) suggest that a large portion of the exposed polypeptide chain (over 80% in MHr) may be of significantly high mobility when proper corrections are made for the effects of crystal packing (see Figure 1). In proteins showing a pattern of mobility similar to MHr, most randomly selected polypeptides would include relatively mobile areas. On the other hand, information on the atomic mobility of a protein in solution is more difficult to obtain from crystallographic data for proteins such as insulin and TMV, in which crystal packing contacts involve a large proportion of the exposed surface of the subunit. The studies on MHr using specifically designed anti-peptide antibodies employed the highly accurate temperature factors determined by Sheriff et al (35). These are unique in being empirically corrected for the crystal contacts. In general, we find that many areas of low mobility in protein structures may be the result of crystal and subunit packing interactions rather than of their intrinsically high order.

Since an accurate evaluation of site mobility is not available for most proteins, this correlation of antigenicity with site mobility is not expected to have great predictive value, except in those few cases when the refined atomic structure is known. In some instances, a secondary-structure prediction of turns and other nonrepetitive secondary structure in conjunction with hydrophilicity profiles can probably be used to select likely sites of high mobility due to the correlation of these parameters noted previously. Based upon their studies of TMV, Westhof et al (145) argue that the reactivity of anti-peptide antibodies with the native protein is related to the existence of contiguous determinants associated with bends, turns, and loops. In TMV, α helices are mostly buried by subunit assembly into a disk aggregate; this subunit association may have affected the immunoassay results. Since a portion of the TMV domain has an antiparallel helix fold similar to that of MHr, the lack of helical determinants is difficult to understand on the basis of structure alone. Although different types of protein secondary structure might be expected to differ in mobility, the MHr data indicate that the type of secondary structure does not greatly affect the antigenicity of protein sites.

Since tertiary structure interactions are quite important for local mobility, a predictive correlation better than that provided by secondary structure might be based upon the probable high mobility of residues associated with the boundaries between introns and exons. Sprang and his colleagues have found that exon-intron boundaries often associate with relatively low-density packing interactions (149a,b). An inference from this association is that exon-intron boundaries are associated with relatively mobile regions. Therefore knowledge of the genomic DNA sequence and intron-exon positions may provide clues to those seeking mobile antigenic regions when three-dimensional structural information is not available. For proteins of known three-dimensional structure without

available mobility data, mobility can be estimated from molecular shape using the method of Connolly (38, 42); the simplest criterion for high mobility is exposure to a large probe sphere, which is indicative of the most highly convex regions of the surface. When neither the genomic DNA sequence nor the protein structure is known, inferences of local mobility might be made from the use of proteolytic enzymes as probes of protein unfolding (144). In any case, perhaps the most important aspect of the data on site mobility is the increase in our understanding of the molecular basis for antigenicity. Some of the implications of the current data are discussed below.

Limits to Site Mobility

The structural fluctuations of proteins in solution are larger than those suggested by their crystallographic temperature factors. Dynamics calculations show that the conformational flexibility of areas with high temperature factors is significantly greater than that seen in the X-ray crystal structures (10, 11). The mobility predicted by the highest average main-chain temperature factors in proteins approximates displacements between ± 1.0 Å and ± 1.5 Å. Rather than defining exactly the excursions of individual residues, however, these isotropic displacements point to areas of relatively low energy barriers between alternative conformations (9, 150, 151).

From the thermodynamic point of view, a protein is a very small system (152). For a given temperature, the root mean square of the energy fluctuations, δH, of a given molecule can be expressed as $\sqrt{\overline{\delta H^2}} = \sqrt{kMCT^2}$, where k is Boltzmann's constant, M is mass, and C is heat capacity. Since an average protein might have a mass of 5×10^{-20} g and a specific heat capacity of $C = 0.32$ cal degree^{-1} g^{-1} at 37° (153, 154), the fluctuations become 7×10^{-20} cal or about 40 kcal/mole, whereas the ΔG total for protein folding is closer to 10 kcal/mole (155). It is thus the short relaxation times (in the nanosecond range) of the fluctuations rather than their energies that keep protein structure from undergoing substantial unfolding (156). Nevertheless, such fluctuations have been verified experimentally. For example, in bovine pancreatic trypsin inhibitor, the Tyr side chains which are rigidly encapsulated in the X-ray structure are clearly flip-flopping about the $C_\beta C_\gamma$ bond by 180° in NMR experiments (157, 158). NMR has similarly verified conformation mobility in lysozyme (159), ribonuclcase (160), myoglobin (161), and cytochrome *c* (82).

Affinity of Antigen-Antibody Interactions

It is important to put the data correlating site mobility and antigenicity in the perspective of biological action. Is mobility essential or incidental to

antigenic recognition? Or in more specific terms, what theories can be devised to establish a relation between them? Consider the energy of antibody-antigen interaction with respect to the flexibility of the antigen. There is a balance between the advantages of mobility in regard to improved stereochemical complementarity and the disadvantages due to entropy. Antibody binding to a mobile site must involve a reduction of its confirmational entropy at the expense of association energy. Antibody binding to a rigid site, not requiring conformation stabilization, would be stronger than to a flexible site, provided the epitope represents a stereochemically complementary surface. However, it seems reasonable that antibody-antigen interaction would benefit from site mobility, allowing numerous small shifts in position. A disordered site (with a large amount of conformational variation in solution) might allow changes required for complementarity, but would incur a larger cost in affinity due to the reduction in entropy.

Although the segmental mobility of antibody and antigen domains can be relatively large (9), the local flexibility or the mobility within both antibody and antigen domains is likely to be much more limited but nevertheless highly important. Consider, for example, the energy cost of hydrophobic gaps in a binding surface. Such gaps can be modeled by comparison to a hole in a nonpolar liquid in which the energy required to form a gap is equal to the area of the gap multiplied by the surface tension of the liquid. Assuming a surface tension of 28 ergs/cm^2 (benzene at 25°C), the energetic cost of a gap is 40 calories/Å^2. A small gap of 40 Å^2 area would cost 1.6 kcal, or more than an order of magnitude, in binding affinity. Although a few such gaps are possible for a tight antibody-antigen interaction, several gaps significantly reduce the binding energy and hence the effective antigenicity. Hydrophilic interactions are similarly dependent upon the formation of appropriate stereochemical complementarity. Hydrogen bonds and salt links lose energy with distance, and in the case of hydrogen bonds the angle of interaction is also quite important (162). Obviously, reduction of the hydrophobic and hydrophilic gaps is energetically favored only if the required conformational changes do not involve breaking too many other favorable interactions, i.e. are not more expensive energetically. These arguments suggest that local flexibility may be essential to antigenic recognition.

Coinciding with the above theoretical discussion, experimental studies also suggest that antibody-antigen interaction does not, in general, cause major rearrangements of the antigen. Single crystal studies of Fab lysozyme complex at 6 Å resolution indicate that no gross changes occur in the antigen structure (146, 147). The data from MHr suggest that there are also limits on the conformational flexibility involved in the reactivity of anti-

peptide antibodies with the intact protein (32). One way to examine experimentally the degree of unfolding involved in the recognition of antigenic sites by representative anti-peptide antibodies is to look for recognition of the same sequence in different protein structures. A number of six and seven residue sequences have different backbone conformations in two known protein structures (163). Antigenicity studies in progress by Wilson and his colleagues (163) will determine whether anti-peptide antibodies can recognize the same sequence in two different conformations. The combination of these crystallographic and immunological experimental studies promises to provide additional information relating site mobility and antibody-antigen interaction.

Mobility As An Antigenic Bias

Mobility is unlikely to be the only intrinsic factor involved in determining the degree of antigenicity. For example, molecular surface topography may also play an important role. However, like the parameters of exposed area and hydrophilicity, surface shape is highly correlated with local mobility. Of course, certain sites on a molecule may not elicit an immune response even though they are flexible, if B cells reactive to these regions are not present as a result of immunological tolerance (54, 55) or of possible evolutionary effects on the germ line repertoire (77). These regulatory factors are particularly pertinent to protein antigens from mammalian sources that have a high degree of sequence homology with host protein, e.g. myoglobin, cytochrome *c*, insulin, and bovine pancreatic ribonuclease, but they may also be important for lysozyme and lactate dehydrogenase, for which lesser homology exists. In retrospect, the correlation of mobility and antigenicity appears to be a reasonable consequence of antibody function. Enzymes have evolved for optimal interaction with a specific substrate. In contrast, antibodies must balance the requirement of an economy of genetic information in providing for recognition of all possible foreign antigens with that of appropriate specificity to protect against inappropriate cross-reactivity.

The current data suggests that limited local flexibility may allow these opposing requirements to be reconciled. It is important to realize that there is no evidence that it is impossible to make antibodies against highly ordered sites in proteins. Rather there is good evidence that antigenic recognition preferentially involves flexible sites. However, the realization that mobility is a parameter affecting antigenicity has implications for any model of antigenic recognition in that it implies a multiple-step process, rather than single collision resulting in simple lock-and-key complementarity. Presently, antigenic recognition can be considered as consisting of at least three stages: (*a*) precollision orientation by electrostatic forces, (*b*)

local antibody-antigen recognition involving the interaction of specific residues, and (*c*) the induced fit accomplished by local rearrangements of epitope and paratope structure.

SUMMARY AND IMPLICATIONS

The ability of anti-peptide antibodies to recognize their corresponding intact folded proteins is best understood as a dynamic process that is most favored when (*a*) the conformation of the peptide immunogen approximates the target site in the protein, and (*b*) that site is mobile. The relative contributions of each factor to immunogenicity are at present difficult to assess and, indeed, may vary from peptide to peptide. The data from both anti-peptide antibodies and from mapping protein epitopes suggest that antigenic recognition in general involves the interaction of antibodies with sites of the protein that can adopt multiple conformations distinct from the average structure seen by X-ray crystallography. Thus, we may envision a process whereby the initial interaction with the antibody is sufficient for recognition of the appropriate site, after which significant induction of shape may take place. In the case of anti-peptide antibodies, the closer the conformation of a peptide approximates that of the site in the protein, the less induction is necessary, and vice versa. In these terms, mobility is involved in both processes: to adopt protein conformations suitable for recognition by the antibody, and to allow induction of a given region into a conformation that fits the antibody binding site.

Besides playing a role in antigen-antibody recognition, differential mobility may modulate conformational changes induced in the immunogen in vivo by emulsification with the adjuvant and by interaction with lymphocytes; this question needs further study. Currently, the anti-protein reactivity of antibodies (raised against synthetic peptide antigens) correlates better with the mobility of the target sites than with their exposed surface area. Furthermore, this correlation of mobility with antigenicity is supported by retrospective studies of the antigenic sites in known protein structures. Thus, the apparent relationship between exposed area and antigenic reactivity (3) may actually reflect the increased mobilities of turns, loops, and other highly exposed areas.

Protein-protein interaction is a complex process in which multiple characteristics of the molecular surface play a role. Both shape complementarity and hydrophobicity of the interacting molecular surfaces are known to be major factors in protein-protein recognition (164). The mapping of electrostatic potential onto molecular surfaces (31, 165, 166) has revealed an important role for electrostatic forces in intermolecular interactions. The results presented here add another dimension to our understanding of

protein-protein interaction: The correlation between the mobility of sites in a protein and their reactivity with antibodies suggests that knowledge of molecular mobility is crucial to understanding the process of antigenic recognition and perhaps to protein-protein recognition in general. Knowledge of the amino acid sequence of a protein should allow the use of site-specific anti-peptide antibodies for the investigation of the role of conformational flexibility in protein-protein recognition processes.

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CONTENTS

Ann. Rev. Immunol. 1985. 3 : 537–60

MOLECULAR GENETICS OF THE T CELL–RECEPTOR BETA CHAIN

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INTRODUCTION

T-cell recognition has long been one of the unsolved mysteries of immunology. The object of a number of recent reviews (1–3) and the occasional polemic (4), this subject has been among the most intensely studied in the entire field. Starting with the introduction of cell cloning and hybridoma technologies as reviewed in Fathman & Frelinger (5) and the more recent discovery of idiotype-bearing, heterodimeric structures (6–11), that have both variable and constant peptide components (12–13), these efforts have been of critical importance in any approach to this problem. This article will attempt to summarize the recent work on the beta chain genes of mouse and human and will try to place this data in its proper biologic context. In the last section, I will attempt to deal with evidence pertaining to genes encoding other chain(s), but this data is just now becoming available and thus a thorough treatment is not possible at this time.

As a departure point for this manuscript, it is useful to summarize the available data on T-cell recognition at least as it pertains to T helper and T-cytotoxic T-cell subsets. These can be divided into immunoglobulin-like and nonimmunoglobulin-like properties. In this first category, it has been established that T lymphocytes, like B lymphocytes, have an extremely diverse repertoire of specificities—easily many thousands and quite likely many millions of possible combinations (14–16). Second, also like B lymphocytes, T-cell specificities are clonally distributed, thus, they are fixed into a given T-cell lineage (5). These immunoglobulin-like properties, which

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led to so much confusion and so many erroneous assumptions in initial studies (as reviewed in 17), are now indeed explainable in terms of the strikingly similar types of molecules and mechanisms employed by the T cell–receptor genes and by immunoglobulins; these will be detailed in this review. Those features largely unique to T-cell recognition are: (*a*) the inability to bind to free antigen alone and the need to see antigen and self-MHC (major histocompatibility complex) determinants together (18–20), and (*b*) the predisposition towards self-MHC, somehow learned prior to antigenic exposure, probably in the thymus (21, 22). These last principles of T-cell recognition are the most difficult to envisage and will no doubt require some years before they are properly understood. Knowledge of the structure and patterns of expression of the various T cell–receptor genes and the manipulation of these through recombinant-DNA technologies should greatly aid this understanding.

ISOLATION OF cDNA CLONES ENCODING T CELL–RECEPTOR POLYPEPTIDES

The isolation of T cell–receptor cDNA clones in both mouse and human systems has been unusual—not, with one exception, directly dependent on the protein work, either through a translational assay or through oligonucleotide probes made from amino acid sequence. Instead, the initial isolation and characterization of candidate clones has rested on differential or subtractive cDNA hybridization schemes and on analogies to immunoglobulin. The first element in either strategy derives from the fact that T cells and B cells differ in only a small fraction of their gene expression—2% (23, and unpublished observations). Thus, by screening for cDNA clones specific to T cells, one could hope to enrich substantially (50×) for T cell receptor–encoding molecules. The relative merits of using differential versus subtractive cDNA hybridization have been reviewed at length elsewhere (17). It appears from that analysis and several examples in the literature (24–29) that the various forms of subtractive hybridization seem to be a more attractive route, particularly for species of modest abundance, although obviously both methods have been successful in various systems, including the one discussed here.

In the case of the human T cell–specific cDNA clone, the first indication that it may encode a receptor molecule was the significant homology of the predicted amino acid sequence to the immunoglobulin variable (V), constant (C), and joining (J) regions (30). In the mouse example (26, 31), a series of T cell–specific cDNA clones were surveyed for rearrangement in different T-cell lines, and the one clone that was positive in this assay was shown to have different V and J segments when a number of homologous

clones from a thymocyte cDNA library were surveyed. Subsequently, the human cDNA clone was also shown to detect rearrangements amongst T-cell lines (32, 33), and N-terminal amino acid sequencing indicated that both the murine and human cDNA clones encoded the beta chain of the typical heterodimeric structure (34).

GENOMIC ORGANIZATION

The isolation and characterization of murine genomic clones have indicated the sequence organization depicted in Figure 1 (35–39). The human beta-chain locus is very similar except that there appear to be fewer J-region elements in the first (most 5′) cluster (T. Mak, personal communication). As noted previously (36), this organization is a curious mixture of the J_1-C_1, J_2-C_2 organization of the murine lambda and the J_1-J_4 organization of the heavy-chain locus. Rearrangement occurs indistinguishably from Ig, where a V region and its 5′ flanking sequences join with a D element and J region to form a discrete V exon separated by a 3 kilobase intervening sequence from the constant region. In several cases, the same V can join to either J-region cluster, indicating what may be the general rule (40). However, usage of each cluster does not appear random, as will be discussed in a later section.

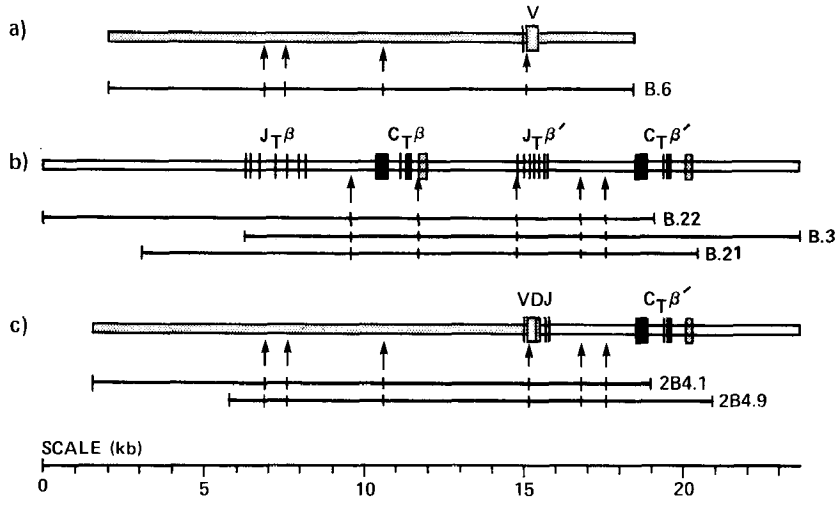

Figure 1 Organization of the murine T cell–receptor β-chain locus. D, J, and C region elements are indicated and compared to the analogous immunoglobulin heavy- and light-chain genes.

CHROMOSOMAL LOCALIZATION

Analysis of somatic cell–hybrid panels and in situ hybridizations have shown that the murine–beta chain locus is on chromosome 6, relatively close to the murine–kappa gene family (42, 43). Restriction polymorphism mapping that uses recombinant inbred lines places this distance at about 10 centimorgans (cM) (44). In situ hybridization placed it somewhat closer—$\leq$5 cM (42), but both methodologies have substantial errors. This juxtaposition of Igκ and the T cell–receptor beta-chain gene is interesting, but the case of the human mapping, in situ hybridization, and somatic cell hybrids place the gene at chromosome 7 (42, 45). It is not clear which band, since the in situ data slightly favor 7q (42), although there is also substantial hybridization of 7p, which is favored by another group (45) because of a translocation chromosome in a somatic-cell hybrid. In any case, human chromosome 7 does not contain any of the Ig-gene families, and thus there is no obligatory link between these two types of recognition structures. Clearly other species need to be surveyed to see which is the more common pattern.

CONSTANT REGIONS

Each of the two constant regions of the murine beta chain is divided into four nearly identical exons, as indicated in Figure 1 (36, 37). The first consists of a 125–amino acid (aa) external domain, the single globular domain of the constant region. This contains cysteines spaced 61 aa apart which presumably form the characteristic intrachain sulfhydryl linkages of the immunoglobulin-like molecules. There is also a cysteine residue internal to these which seems analogous to those found in the constant region of rabbit kappa–light chain; the residue has been postulated to stabilize the molecule further through a second intrachain sulfhydral bond (46) to a cysteine in the variable region. Although a number of beta-chain V regions have extra cysteines, not all do, and it is questionable what would happen in those cases. It has also been noted that this exon is somewhat larger (by about 16 aa) than Ig domains, and this extra portion has been designated a *connecting peptide* (37).

The second exon is only 6 aa in size and contains the last cysteine of the constant region and presumably forms the sulfhydral bond known to join the alpha and beta chains of the receptor (31). This region has homology both to the area around the last cysteine of lambda–light chain constant region and to one of the heavy chain (gamma 1) hinge regions (31, 36). Its

position as a separate exon is also reminiscent of many of the Ig hinge regions (47). Although it contains no proline residues and thus would not be as flexible as an Ig hinge, it may serve a similar but perhaps more limited function in the T cell–receptor heterodimer. Its status as a separate exon appears highly conserved in evolution, occurring in both of the human constant regions (T. Mak, personal communication) and in genomic clones corresponding to the T-cell receptor-like gene isolated by Saito et al (28) (S. Tonegawa, personal communication).

The third exon is 36 aa, most of which are quite hydrophobic, and thus the exon probably spans the membrane (31). Interestingly, a lysine residue occurs almost in the middle of this exon, apparently disrupting its hydrophobic character. This lysine occurs in both the human and murine sequences, as well as in the sequence of the second type of T cell–receptor gene (28); therefore it must serve some function in either the intrachain stabilization or in the interaction with another protein in the membrane (perhaps one of the T3 subunits). The fourth exon consists of 6 aa, containing a string of three positive charges, typical of a cytoplasmic anchor. These sequences are directly contiguous with the entire 3′ untranslated region, as in most Igs although different from H-2 and Ia genes.

A Comparison

Of immediate interest is comparison of the sequences of these respective $C_T\beta$ exons to determine whether they might encode functionally distinct molecules or isotypes analogous to the immunoglobulin–heavy chain genes. A summary of these comparisons, together with one of the human $C_T\beta$ sequences, is shown in Table 1. All are very similar; in fact the mouse sequences differ by only 4 aa and all of these differences are in the last exons (36). Thus it seems unlikely that these two constant regions could encode functionally different receptors. This data also indicates that the amino acid sequence has been highly conserved; the divergence of the 3′ untranslated region (a generally unselected sequence) indicates that the murine genes duplicated over 120 million years ago, or longer (36), well before mammalian speciation (75 Myr). Also, the homology to the human sequences is overall 80% of the protein and higher in the transmembrane exon (36). The fact that the nucleotide divergences in the first exon are so different between mouse and human appears to be another byproduct of a gene-correction mechanism within a family of genes in a given species. These data combine with a similar (80%) homology to rabbit sequences (R. Mage, personal communication) to indicate that overall, the T-cell receptor is more conserved than any of the immunoglobulins—the latter range from

Table 1 Homologies of constant region exons

	Exon				
	External C_T1	Hinge C_T2	Transmembrane C_T3	Cytoplasmic C_T4	3′UT
Nucleotide changes					
(m/m)[a]	1/375	3/18	16/107	4/18	103/217
(m/h)[b]	74/375	3/18	14/107	4/18	—
Amino acid changes					
(m/m)	0/125	0/6	3/36	1/6	—
(m/h)	26/125	1/6	2/36	2/6	—

[a] m = mouse (Ref. 36).
[b] h = human (Ref. 30).

50–70% in analogous interspecies comparisons (48), although higher in specific portions. It has been suggested (36) that this higher degree of conservation of the constant region results from the apparently large number of other cell-surface molecules with which it may have to interact.

Further attempts to isolate constant regions other than C_T have involved attempts to screen thymocyte libraries for cDNA clones that hybridize to J or V without C. In both cases, positive clones have subsequently turned out to represent incomplete splicing artifacts, although relatively few were characterized completely (Chien et al, unpublished observation). Nor have any functional helper- or cytotoxic-cell lines been found that fail to utilize the $C_T\beta$ locus described here; this further indicates that isotype switching—at least as it is found in the Ig case—is not operating here. Therefore it may be that isotypic functions are manifested in separate molecules such as L3T4 and Lyt 2, as has been suggested (49, 50). Indications are that isotype switching is relatively recent in evolution, first appearing in the bony fishes (51), and therefore after the probable date of TCR : Ig divergence.

JOINING (J) REGIONS

One of the most striking features in the organization of the beta-chain locus (Figure 1) is the large number of possible joining (J) regions in the two clusters. In all, six out of the seven J-region elements in each cluster appear usable (35–37). A compilation of 19 cDNA clone sequences, from our own laboratory and from the literature, is shown in Table 2. This indicates that at least 9 of these 12 possible J regions are capable of rearrangement,

although the frequencies of usage are not random. In particular, one J region in each cluster apparently predominates; specifically, J_T3 occurs five times and J_T5' is used six times in the 19 cDNA clones surveyed here. It is also evident from this table that while cDNAs from normal tissue somewhat favor the first locus (4/6), mature T-cell lines and hybrids disproportionately utilize the second (8/10). As previously mentioned, it is not clear why this should be so; it may reflect some bias in the choice of cells studied, but it could be important. There are sufficient sequence differences in the putative recombination signals between the first and second J clusters to allow regulation (see below). It is also evident that T_H cells can use either cluster or constant region, which further indicates the lack of gross functional differences between these sequences.

One important question to ask concerning these J elements is whether they have the conserved seven and nine nucleotide elements believed to be important in Ig variable–region formation (as reviewed in 52). To address this point, the 5′ flanking sequences of all nine usable J regions discussed above are shown in Table 3, and the consensus sequence of each cluster is compared to the Ig consensus sequences. It is clear from this analysis and from that of other beta chain–gene segments (next section), that the T-cell

Table 2 J region usage

cDNA	Source	Phenotype	Specificity	MHC or restricting element	J region	Ref.
86C3	Con A Spl.			d	J_T1	(82)
86S2	Spleen			k+d	J_T1	(40)
86C1	Con A Spl.			d	J_T2	(40)
86T1	Thymus			d	J_T3	(31)
86T5	Thymus			d	J_T3	(31)
86S1	Spleen			b	J_T3	(40)
FN1-18	Hybrid	T_H	KLH		J_T3	(83)
1-3.1	Hybrid	T_H	allo		J_T3	(84)
Ly23,4	Line	T_S			J_T1'	(41)
E1	Line	T_H	TNP	$I\text{-}A^d$	J_T2'	(41)
LB2	Line	T_H	CRBC	b	J_T3'	(41)
86C5	Con A Spl.			d	J_T4'	(40)
2B4.71	Hybrid	T_H	Cyto c	k+d	J_T5'	(35)
BW5147	Lymphoma			k	J_T5'	(40)
C5	Line	T_H	DNP-OVA	$I\text{-}A^b$	J_T5'	(41)
G4	Line	T_C	D^d	b	J_T5'	(85)
TM86	Hybrid	T_H	D-J	b	J_T5'	(31)
86C6	Con A Spl.			d	J_T5'	(40)
2C	Line	TC	D^d	b	J_T7'	(28)

Table 3 J regions and flanking sequences

9-mer	13 nt spacer	7-mer	Coding region	
TATTTTTCT	CCTCATCCTATGG	CACTGTG	NTEVFFGKGTRLTVV	J_T1
CCATATTCG	AGTATCTGTATTC	TGATGTG	NSDYTFGSGTRLLVI	J_T2
GGGTTTTGA	AGTGAACCCGGGA	GGCTGTG	SGNTLYFGEGSRLIVV	J_T3
	11–12 nt spacer			
GAATTCTGG	TAGCCCTTTTC	TGCTGTG	NYAEQFFGPGTRLTVL	J_T1'
GGTTTGTGC	CAGCATTTCCAA	GACTGTG	NTGQLYFGEGSKLTVL	J_T2'
AGTTTTTGT	CCTGAGCCTGGA	GGCTGTG	SAETLYFGSGTRLTVL	J_T3'
AGTTTTTGT	CCTGTACCAAGA	GGCTGTG	SQNTLYFGAGTRLSVL	J_T4'
AGTTTTTGT	GTTGGGTTCTGG	GGCTGTG	NQDTQYFGPGTRLLVL	J_T5'
GGTTTGTGT	GTGGGGTTGAGC	CTCTCTG	SYEQYFGPGTRLTVL	J_T7'
AGTTTTTGT	12 or 23 nt	CACTGTG	FG GT	Ig Consensus Sequences

receptor sequences are very similar to, although not identical to those flanking the Ig elements. Particularly the CTGTG section of the 7-mer and the T-rich interior of the 9-mer are preserved, as well as the 12 or 23 nucleotide spacing between. The significant differences are also clear, especially at the beginning of 7-mer; this may be sufficient to allow some independent regulation both of Ig and TCR and between the clusters. Thus, the different use of two clusters may stem from differences in J region–flanking sequences. An additional point indicated in this table is the relatively variable 5′ portion of the beta-chain J regions; they seem generally more diverse in this region than are the murine immunoglobulin J regions (4, 8).

DIVERSITY (D) REGIONS

Diversity or D elements in immunoglobulin heavy-chain genes were first postulated to exist by Schilling et al (53) based on the independent assortment of amino acid sequences between V and J. This was even more strongly indicated by the work of Early et al (54) who found that a portion of the complete V_{HS107} gene could not be accounted for by the appropriate germline V_H and J_H sequences. Such elements were subsequently identified in genomic DNA by Sakano et al (55) and Kurosawa et al (56). These very small genetic elements, typically contributing only 1–5 amino acids but ranging up to 15, are the closest approximation to mini-genes that Kabat et al earlier suggested might represent the different complementarity-determining regions (CDRs) (57). They encode a significant portion of the third CDR. Through variable joining at either end, apparent joining in different reading frames, as well as the combinatorial properties of an additional genetic component to the V_H genes, they have a significant effect on the available repertoire (52).

Similarly, sequence analysis of rearranged T cell–receptor V regions indicated that some of the nucleotides could not be accounted for by the presumptive germline V and J segments, indicating the involvement of a D element (35, 32). Shortly, thereafter, single D elements were found adjacent to each of the two J-region clusters in both mouse (38, 39) and human (57a) (see Figure 1). These D-region elements are frequently rearranged without V regions and are transcribed, giving rise to a 1.0 kb mRNA species versus the typical 1.3 kb species containing the V region (39, 40). This exactly parallels recent work with D-J joining in immunoglobulins by Alt (personal communication) and Honjo (58a) and their colleagues, that further indicates that D-J joining in Ig is, at least in some cases, a precursor to V-D-J joining. It also seems likely that the D-J-C transcript is translated since in all known cases a hydrophobic peptide of 15–50 aa's precedes the D-J joint and, in the

case of the beta-chain D regions, the sequence of at least one of these putative leader peptides is highly conserved between mouse and human. There appears to be a very high incidence of D-J joinings in T cells, accounting for approximately half of beta-chain mRNA in thymocytes (39), and D-J-C transcripts are often seen in mature cell lines and hybrids.

Our own analysis of the T_H hybridoma, 2B4, indicates that there are two non-BW5147 chromosomes, one containing the functional, rearranged gene (35) and the other having two separate D-J joinings to each of the J clusters by the immediately adjacent D elements (D. Becker, unpublished observation). Furthermore, in a large survey of T-cell lines and hybrids (66), we saw only one line that had an unrearranged chromosome. These data indicate that D-J joining is very common and may be presumed to be a "fast" reaction and that subsequent joining to V is relatively infrequent and perhaps "slow" kinetically. It seems fair also to believe that D-J joints are not always precursors to V-D-J joining, since many stable lines and hybrids have them.

Another important aspect of beta-chain D regions is the fact that the organization of heptamers and nonamers around them and the respective V and J regions does differ significantly from that of heavy chains. Figure 2 summarizes the spacing of these elements around the two loci. In each case, during a V-D-J rearrangement, elements spaced by 12 nucleotides join to elements spaced by 23 nucleotides or vice versa, the so-called "12/23" rule (52, 54). This is another indication of the mechanistic similarity of this process in both immunoglobulins and the T cell–receptor beta chain. However, whereas V-J and D-D joinings are excluded by the 12/23 rule in heavy chain–gene segments, they seem allowable in the beta-chain locus (38, 39). Figure 3 illustrates how this alternative joining event might take place, adding to the flexibility of the variable region. Thus far, one potential

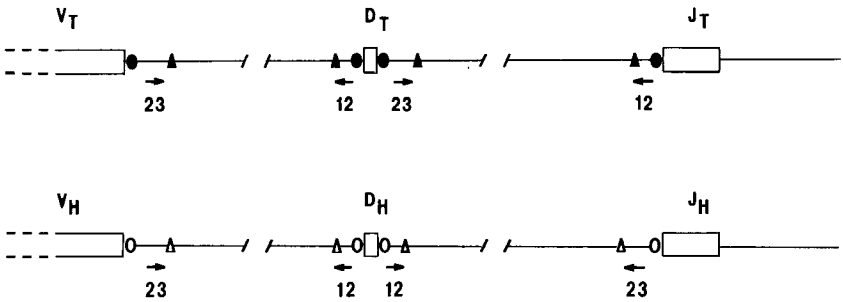

Figure 2 Heptamer and nonamer organization of immunoglobulin–heavy chain genes and the β chain. Heptamers are indicated by circles and nonamers by triangles. Spacing is either 12 or 23 nucleotides. Arrows indicate the orientation of the elements.

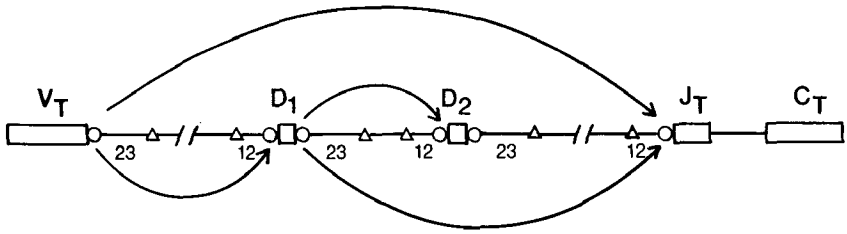

Figure 3 Models of V-D-J joining in the β chain gene. Arrows above the line indicate orthodox V-J-J joining processes, those below the speculative V-J or D-D rearrangements that may take place.

case of direct V-J joining has been found in human beta chain (58, 58a), although this may be a relatively rare event since we have not seen it in approximately 15 V-D-J sequences in the mouse. There has also been one possible D-D joining (in the mouse) (40), although other interpretations are possible.

VARIABLE REGION STRUCTURE

As with the other beta-chain gene segments, the variable regions are of very similar size, and portions of them share sequence homology with those of immunoglobulin-V regions. Overall, they are about 20% homologous at the protein level to selected Ig-V regions; short stretches can be virtually identical. An example of this is the 11/12 amino acid homology found around the last cysteine of the 86T1/variable region with an antiarsonate (93CL′) heavy chain–variable region (31). Such homologies have led to the speculation that the apparent cross-reactivity of beta-Ig-idiotypic serums (31) could be explained in terms of similar V-region epitopes. A compilation of the seven V_TB sequences available to date (28, 30, 31, 35, 41) is shown in Figure 4 (from 41). It is not yet clear where the exact posttranslational cleavage of the murine beta chain is. Homology to Ig's (31) originally suggested the processed peptides shown in Figure 4, but recent N-terminal amino acid from a human protein identical in sequence to YT35 (34) indicates that cleavage is two aa's further into the V sequence. Sequence analysis of a murine beta chain may be the only way to resolve this uncertainty. Given the putative sequence as shown, however, the V_β's range from 93–96 complete aa codons in length and end two nucleotides into an adjacent codon.

A notable feature of the homologies shown in Figure 4 is how little amino acid sequence most $V_T\beta$'s have in common. They range from 18–51% homologous (41) versus 45–98% homology among Ig V regions of a given

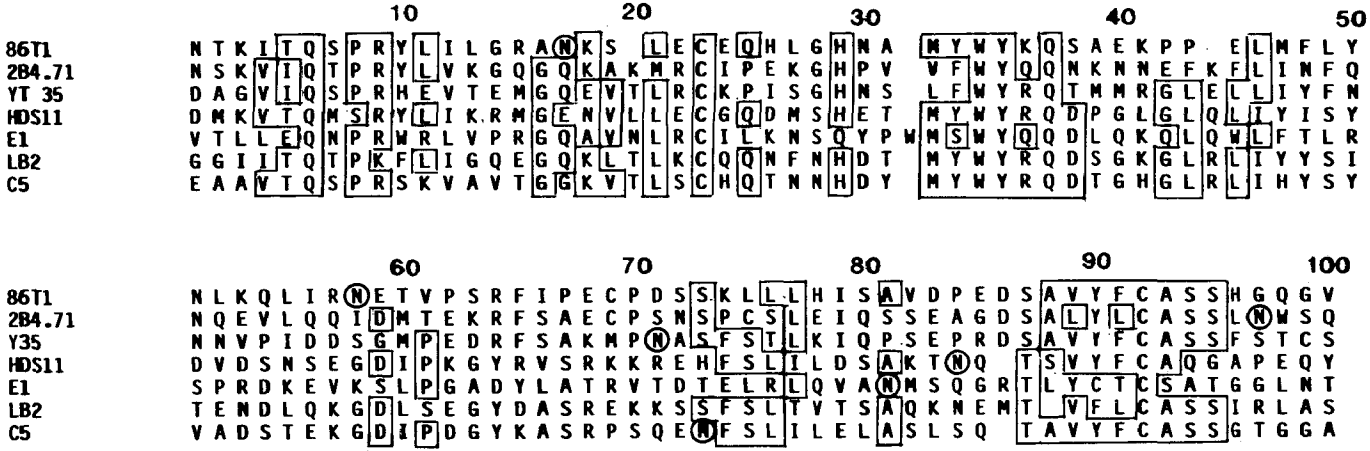

Figure 4 Comparisons of V_β sequences (41). 86T1 is from ref. 31, 2B4.71 from ref. 35, YT35 from ref. 30, HDS11, ref. 28, E1, LB2 and C5 are from ref. 41. Boxes indicate homologies and circles indicate N-linked glycosylation sites (N-X-T/S).

family (48). A further indication of this wide sequence divergence is that most $V_T\beta$'s in mouse (41) and human (T. Honjo, personal communication) hybridize to only one or two bands on a genomic southern blot at moderate stringency. This compares to the 4–30 bands seen with most $V_H + V_K$ probes (59, 60) and indicates that if there are families of V_β regions, they have diverged much further from each other than members of the Ig V–region families (41). One explanation of this greater divergence is that the T cell–receptor genes preceded Ig in evolution, since cellular immunity appears to precede humoral immunity (51). While this is quite likely the case, there is now strong evidence that an additional factor plays a role in the lack of homology between V_β's; namely, there is evidence for as many as four additional hypervariable regions in these sequences (41). These analyses follow the pioneering work of Kabat & Wu (61) who first proposed that hypervariable residues in immunoglobulin formed the binding site of that molecule. They found thus three hypervariable regions—in both heavy and light chains—that were later shown, by X-ray diffraction analysis of protein crystals (62–64), to be involved in forming the antigen-combining site of the Ab molecule. In an attempt to define the hypervariable and framework regions of $V_T\beta$'s, a variability plot of the seven V_TB sequences shown in Figure 4 was computed by the formula: (number of aa's)/(fraction of the most abundant aa) (61).

The result is shown in Figure 5 (41) and indicates that there may be as many as seven significantly hypervariable peaks in V_β, three at positions similar to those of Ig (shown by arrows) but four at entirely new positions. The three additional V_β sequences reinforce this conclusion (G. K. Sim; N. Gascoigne, J. Elliot; N. Lee; all personal communications). In order to be sure that this result was not an artifact of the relatively small number of sequences available, Patten et al (41) also examined homologies between a small number of V_κ's and V_H's (Figure 5b) and found that even though these sequences were chosen to be fairly remote from each other (from different subgroups or species) they were still fairly accurate at predicting framework regions (as underlined). By comparison, the V_β homologies, while similar to Ig in a few portions, had large stretches of nonhomology, coinciding with the proposed new hypervariable regions. Such a large number of hypervariable regions would increase the divergence rate of the V_β gene segments since in Ig it has been observed that hypervariable regions are generally unconserved, while framework portions are much more constrained (65). From Figure 5b it can be seen that whereas only about 25% of the Ig V regions consist of hypervariable portions, more than 55% of the V_β's could be so labeled. This difference would have a dramatic effect on divergence rates for the whole segment, thus explaining both lack of crossreactive gene families in most cases and

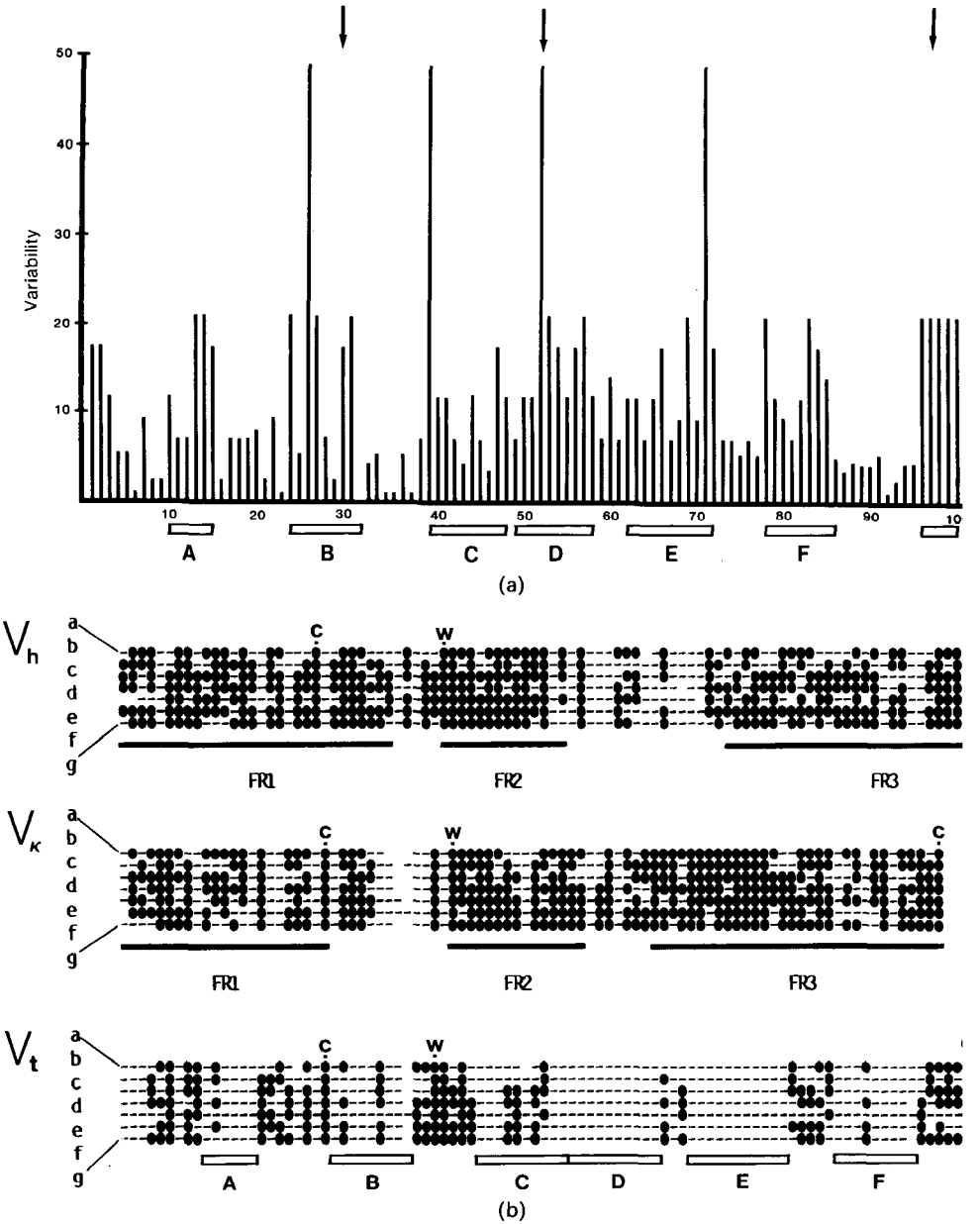

Figure 5 Hypervariable and framework regions of V_β. (*a*) Indicates a Kabat-Wu pot or variability, with arrows denoting peaks of variability found in Kappa–light chain sequences. Horizontal boxes indicate the predicted hypervariable regions. (*b*) Homology comparisons of seven immunoglobulin–heavy chain, amino acid sequences, seven Kappa–light chain sequences—all chosen to be as different as possible (see 41) and the seven V_β sequences. Four out of seven or better is indicated by a closed circle, less by a dash.

the observation that V_β's appear generally less conserved between mammalian species than do Ig V's (41).

Interestingly, when one asks where on the Ig third structure these new hypervariable regions would be located, it seems that most of them must be on the outside of the Ig binding site as shown in Figure 6 (41), leading to the suggestion that important interactions can occur on the side of the variable region as it binds to something, perhaps with MHC determinants.

VARIABLE REGION USAGE

Because of the divergence between V_β's indicated in the preceding section, it is not possible at the present time to estimate how many different V-region genes there are. Approximately ten distinct murine sequences are known, but in principle there could be hundreds or thousands. One way to address this question is to ask how often the average V_β is utilized in normal T-cell populations and then extrapolate to the minimum number of V_β's required.

The results of such an experiment are shown in Table 4, where five different V_β probes (86T1, 2B4.71, C5, E1 and CB-2) were used to screen a thymocyte cDNA library (41). Four of these five probes were present at very high levels: 5–15% of the estimated V-bearing beta-chain cDNA clones. The fifth 2B4.71 was undetectable in this and a variety of other libraries, indicating much lower frequency in unprimed animals. This makes some sense since the V region was derived from a cytochrome-reactive T cell, which represents a very rare response (R. Schwartz, personal communication). These data indicate that a few (5–10) V_β predominate amongst unprimed normal T cells and that there are some unknown numbers of V_β such as 2B4.71 that occur at a much lower frequency. This is a rather unexpected result and must significantly skew the available T-cell repertoire in the organism, although to what purpose is unclear. This class of common

Table 4 Variable region usage in a BALB/c thymocyte cDNA library (41)

Variable region	Frequency	Corrected frequency[a]
C5	3.2%	16%
E1	2.2%	11%
LB2	0.9%	4.5%
86T1	2.0%	10%
2B4.71	<0.05%	
Total	8.3%	41.5%

[a] Frequency divided by the fraction of $C_T B$ positive cDNA clones bearing V regions (0.2).

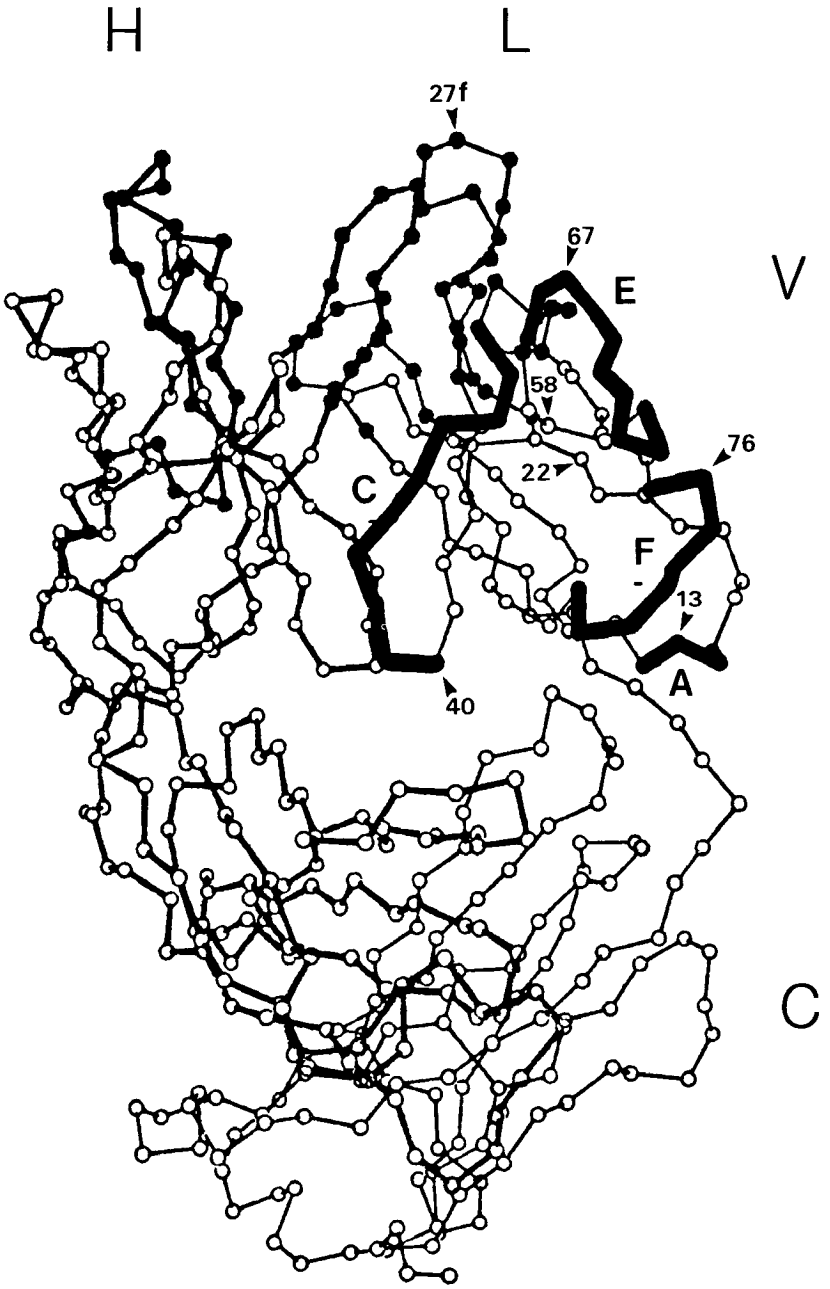

Figure 6 Immunoglobulin structure showing putative new regions of hypervariability. Alpha carbon skeleton from reference 64 of MPC603 structure. Filled circles indicate classical hypervariable regions, thick lines the apparent new regions as indicated by the V_β sequences.

V regions might also explain the result that V_β sequences from three different human T-cell tumors are identical. [MOLT 3 (30); REX, E. Remhertz, personal communication; Jurkat (32).] In fact, the work of N. E. Lee et al (in progress) on a series of eight different AKR thymomas indicates that at least six use V_β genes from the predominant class of V regions, arguing against any specific selection mechanisms.

T-CELL SUBSETS

As mentioned previously, it is clear that all helper and cytotoxic T cells examined utilize the $C_T\beta$ locus, the same J + C elements. There is even a case now where the same V region is used for a helper T-cell line directed against chicken red blood cells (b haplotype restricted) (41) and for a cytotoxic line (G4 that derived from a Balb.b animal is specific for the D^d allele (S. Hedrick, personal communication). This indicates that there is at least some overlap in the V-region repertoires used for both types of cells. The data regarding T_c and T_H are summarized in Table 5 and are true for both mouse and human (33, 66, 67). Curiously, a large number (at least 21) of murine suppressor–T cell hybridomas do not appear to have a functional rearrangement of the beta-chain (66) locus. In many cases they have deleted this locus, as least as far distal to the centromere on chromosome 6 as the kappa–light chain gene (10 cM), and they have probably lost all but the BW5147 alleles (66). Therefore, even though most of these lines were tested for various antigen specific–suppressor activities just before DNA was extracted, these specificities could not result from the donor cell–beta chain, and that of BW5147 appears defective (40). In contrast to this are the findings of groups working with human suppressor cell lines (33, 67), all of which (Table 5) appear to rearrange the beta-chain locus and transcribe a functional message. This apparent discrepancy may relate to the different assays used in the mouse and human systems, but the example of the mouse at least leaves open the possibility that some other loci may be important in particular T-cell subsets.

Table 5 $C_T\beta$ rearrangement or mRNA expression in functional T-cell lines and hybrids

	T helpers	T cytotoxic	T suppressors
Murine lines	7/7	5/5	1/1
Murine hybridomas	15/15	2/2	3/22
Human lines	5/5	7/7	4/4

SOMATIC MUTATION

Studies of both heavy- (68–71) and light-chain (72, 73) immunoglobulins have indicated that somatic mutation, confined to the variable region exon and its immediate flanking sequences, plays a definite role in the generation of antibody diversity. This type of mutation may be an integral part of the secondary response (68), and specific "hypermutation" mechanisms have been proposed (70, 71). It is not yet clear what role, if any, somatic mutation plays in diversifying the beta-chain repertoire. A survey was made of seven cDNA clones bearing the same V region, most of which came from concanavalin A (con A)-stimulated or -unstimulated spleen cDNA libraries from several MHC haplotypes. The survey revealed a total of only two nucleotide changes from the apparent germline sequence (40). Consistent with this result is the finding that a cytochrome c–specific hybridoma, which represents a secondary T-cell response, has no nucleotide changes in its expressed V_β gene segment versus the germline sequence (35). A second cytochrome c–specific hybrid sequence has only one nucleotide change (S. Hedrick, personal communication). In apparent contrast to this are the data of Siu et al (32) who found nine nucleotide differences in a human V_β sequence with a genomic counterpart. However, in this last case subsequent analysis has revealed a second V_β genomic sequence which perfectly matches the expressed sequence (L. Hood, personal communication). Although these results are not conclusive in ruling out a possible role for somatic mutation in the beta chain, they do argue against one of the models of Jerne (74) that postulated a fairly severe and obligatory somatic mutation of self-receptors in order to derive antigen receptors. In addition, it is conceptually reasonable that somatic mutation may not be a major feature of T cell–receptor diversity. This follows from the argument that if thymic selection is designed, in part, to remove self-reactive T cells, then this purpose may be negated by further mutation outside the thymus as part of a secondary response. Immunoglobulins may not be as constrained in this way because they are not as attuned to self-MHC components as are T cells. A corollary to this idea is that some autoimmune disorders may result from the random mutation of a particular T-cell clone. (See also 74a.)

EXPRESSION DURING THYMIC DIFFERENTIATION

All major thymic subpopulations seem to express functional beta-chain mRNA, even very early Lyt 2^-, L3T4$^-$, Thy 1^+ subpopulations (L. Samelson, personal communication). This does not rule out the possibility that small subpopulations or members of a given subset may be negative,

however. Alternative approaches using hybridomas made from different thymocyte subpopulations (L. Samelson, personal communication) indicate that at least some beta chain DNA rearrangements are still occurring in the thymus. Furthermore, hybrids from very early fetal thymocytes that have no rearrangements in the beta-chain locus (P. Marrack, personal communication) are an even stronger indication of this. Even though most thymocytes seem to be expressing the beta chain, it is somewhat puzzling why the majority of cells appear to lack T cell–receptor protein on their surface—perhaps only 15% by some estimates made with human material (12). One factor that has been suggested (38) is that with three gene segments making up the V region in the beta chain (V-D-J), the possibility of out-of-frame joinings may be very high. Perhaps as many as 90% of the V-D-J rearrangements would be unsuccessful on any given chromosome, producing a large number of T cell–receptor negative thymocytes. A second possibility is that the production of the alpha chain is limiting and that alpha-chain rearrangement may occur after that of the beta chain. This possibility is suggested from the analysis of an alpha-chain cDNA clone (75) that showed that the ratio of α to β changed from 1 : 20 to 1 : 3 in thymus and Con A spleen cDNA libraries respectively (76). Even stronger support of this possibility is provided by the result that the "double-negative" ($L3T4^-$, $Lyt2^-$) very early thymocyte population mentioned above expresses normal amounts of beta chain but almost no alpha chain mRNA (T. Lindsten and L. Samelson, work in progress). This also indicates that we may be able to define a true "pre-T" cell stage as being $\beta^+\alpha^\circ$, analogous to the pre-B cell stage of B-lymphocytes.

OTHER CHAINS OF THE T-CELL RECEPTOR

T cell–receptor biochemistry has consistently indicated, in both mouse and human systems, that there are two principal subunits to the receptor α and β, that are disulphide-linked and precipitable with anti-idiotypic antibodies (7–11), anticonstant region serums (76), or anti-T3 antibodies (77). After the isolation of the beta chain cDNA clones, Saito et al (28), using subtractive cDNA methodology (26) on murine cytotoxic T cells, isolated a new, immunoglobulin-like T cell–specific gene (HDS4) that has V, J, and C regions and rearranges in a number of T cells. Although the authors speculated that this encoded the alpha chain, the lack of N-linked glycosylation sites in the sequence of this gene, versus the 3–4 that have been characterized in a number of alpha chain proteins (78–80), and the low level of expression vis a vis the beta chain in a spleen cell cDNA library (1/50) (75), cast some doubt on this possibility. Recently two groups (75, 81) have reported the isolation of another distinct type of immunoglobulin-like, T-

cell gene that seems more likely to encode the alpha chain. The predicted protein has 3–4 N-linked glycosylation sites and is present at frequencies similar to the beta chain in both helper and cytotoxic T-cell cDNA libraries (75). The constant region of this molecule is shared between helper and cytotoxic T cells (75, 81) and it has been shown to encode the authentic alpha chain, by protein sequence analysis of the human (82, 83) and mouse (C. Hannum, personal communication) equivalents (84). This still leaves the question about the function of the HDS4 chain. It may serve as an alternative partner to either α or β or it may combine with yet a fourth molecule. Regardless, it is likely to be specific for some role in the process of T-cell recognition and function, although perhaps confined to cytotoxic T cells, since many helper lines do not express the HDS4 molecule (S. Tonegawa, personal communication, 75).

SUMMARY

Characterization of cDNA and genomic clones encoding the Beta chain of the T-cell receptor for antigen reveals a very close resemblance to immunoglobulin: V, D, J, and C elements; the mechanism of rearrangement; and the potential extent of diversity, explaining the relatively large T-cell repertoire of specificities and the clonal nature of individual responses. Differences with immunoglobulins are evident in the much more heterogenous V_β sequences, which appear to have additional hypervariable regions. Together these data predict a roughly immunoglobulin-like structure for the receptor, but with potentially significant variation from immunoglobulin in the nature of the combining site(s).

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CONTENTS