ADVANCES IN

IMMUNOLOGY

VOLUME 38

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Immunology

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ADVANCES IN
Immunology

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VOLUME 38

1986

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
Orlando San Diego New York Austin
London Montreal Sydney Tokyo Toronto

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 61-17057

ISBN 0–12–022438–0

PRINTED IN THE UNITED STATES OF AMERICA

86 87 88 89 9 8 7 6 5 4 3 2 1

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ADVANCES IN IMMUNOLOGY, VOL. 38

The Antigen-Specific, Major Histocompatibility Complex-Restricted Receptor on T Cells

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I. Introduction

The receptor on T cells responsible for clonal recognition of and response to antigen by these cells has been long sought, not only because of the insights an understanding of these molecules might shed on human health and disease, but also because of scientific curiosity about the way in which such molecules might function. Early attempts to isolate these proteins relied heavily on the idea that T cell receptors might be similar, if not identical, to immunoglobulin. In retrospect although this idea was not unreasonable, it certainly created a good deal of confusion in the field.

Thus, conflicting data were generated about the presence on T cells, or T cells' secretion, of proteins cross-reacting with anti-immunoglobulin antibodies (Marchalonis and Cone, 1973; Vitetta *et al.*, 1973; Eichmann and Rajewsky, 1975; Binz and Wigzell, 1977; Ben Neriah *et al.*, 1980). Similarly, attempts to pursue T cell receptors based on the assumption that, like antibody molecules, they would bind free antigen molecules, also led to opposing results (Hunter *et al.*, 1972; Feldmann, 1972; Binz and Wigzell, 1975;

Krawinkel *et al.*, 1977). Meanwhile data accumulated which suggested that the receptors for antigen on most T cells might not be immunoglobulins.

Perhaps the first indications of this came from experiments which showed that T cells and B cells from the same animals did not recognize the same determinants on antigens. For example, many experiments showed that T cells do not distinguish between native and denatured antigens, whereas antibody commonly does (Parish, 1971; Mason *et al.*, 1972; Schirrmacher and Wigzell, 1972; Chesnut *et al.*, 1980). Moreover, T cells and B cells primed to a particular antigen frequently cross-react differently on related antigens (Hoffmann and Kappler, 1972; Playfair, 1972), although exceptions to this rule were also noted (Rajewsky and Mohr, 1974). In addition, the confusing phenomena of immune response (Ir) genes also had to be explained, since these major histocompatibility complex (MHC)-associated genes were known to affect T cell reactivity without much direct effect on B cells (Katz *et al.*, 1973b; Press and McDevitt, 1977).

The explanation for these findings was founded on the work in the early 1970s of Kindred and Shreffler, Katz and his colleagues, and Shevach and Rosenthal (Kindred and Shreffler, 1972; Katz *et al.*, 1973a,b; Rosenthal and Shevach, 1973; Shevach and Rosenthal, 1973). Later Zinkernagel and Doherty and others built on the findings of these groups, and demonstrated directly for the first time that cytotoxic T cells only kill antigen-bearing cells if the antigen is presented to them in association with products of the MHC on the surfaces of antigen presenting cells (Zinkernagel and Doherty, 1975; Shearer *et al.*, 1975; Gordon *et al.*, 1975; Bevan, 1975). Numerous workers have since confirmed this finding for T cells responsible for B cell help, delayed hypersensitivity, and lymphokine secretion (Kappler and Marrack, 1976, 1977; Singer *et al.*, 1978; Sprent, 1978; Schwartz *et al.*, 1978; Waldmann, 1978; von Boehmer and Haas, 1981).

The discovery that many T cells recognize antigen only in association with products of the MHC led to the conclusion that at least a component of the receptor on T cells was not immunoglobulin. This, coupled with numerous observations that many T cells do not secrete immunoglobulins (Roehm *et al.*, 1984b) or usually have mRNA encoding these molecules (Kronenberg *et al.*, 1980, 1983; Kurosawa *et al.*, 1981), suggested that the isolation of T cell receptors might be most easily accomplished by methods which did not depend on the idea that these molecules were related to immunoglobulins.

In fact the two methods which were most successful in identifying receptors were both based on a minimum number of assumptions. The first experiments depended on the idea that receptors would bear antigenic determinants which would not necessarily cross-react between different T cell clones, and the second was based on the assumption that receptor mRNA would be transcribed only in T cells.

II. Identification and Properties of T Cell Receptor Proteins

A number of investigators identified molecules of interest on T cells by injecting bulk, uncloned T cells or T cell tumors from one strain or species into other animals and studying the properties of the antisera and/or monoclonal antibodies so identified. By these means many important proteins on the surfaces of T cells were identified, some of which appeared to have a role in T cell recognition of antigen, because antibodies against them either blocked or stimulated T cell activation (Itakura *et al.*, 1972; Cantor and Boyse, 1975; Shiku *et al.*, 1976; Nakayama *et al.*, 1979; Davingnon *et al.*, 1981; Swain, 1981; Biddison *et al.*, 1982; Reinherz *et al.*, 1982). Since none of these molecules appeared to vary in amino acid sequence between one T cell clone and another, however, it was concluded that they did not contribute to specific recognition of antigen and MHC by T cells. These molecules are of great interest, however. Some of their properties are discussed in detail below.

A major advance occurred with the description by Infante *et al.* (1982) of antisera raised in mice against alloreactive T cell clones. These antisera stimulated division of the immunizing T cell clone and had no effect on other, closely related T cells, suggesting that the functional antibodies involved were recognizing some clone-specific determinant on the surface of the T cell clone used for immunization, a determinant which might be the analog for T cell receptors of an immunoglobulin idiotype. This work was followed by the description of other antisera with similar properties, and finally by reports from a number of laboratories of monoclonal antibodies which recognized clone-specific determinants bourne by the T cell clones, tumors, or hybridomas used for immunization (Allison *et al.*, 1982; White *et al.*, 1983; Meuer *et al.*, 1983b; Haskins *et al.*, 1983; Samelson and Schwartz, 1983; Kaye *et al.*, 1983; Bigler *et al.*, 1983; Lancki *et al.*, 1983; Staerz *et al.*, 1984; Kranz *et al.*, 1984).

Many properties of these monoclonal antibodies indicated that they bound receptors on T cells. First, their specificity for particular T cell clones showed, of course, that the molecule bound had determinants that were unique to a particular clone. Of all T cell surface proteins, only the receptor for antigen and MHC would have this characteristic. Second, these antibodies inhibited the responses or binding to antigen plus MHC of target T cell clones (Haskins *et al.*, 1983). Alternatively, under certain circumstances the antibodies stimulated responses of target T cells. This was particularly apparent if the antibodies were made polyvalent, by coupling to Sepharose beads for example (Kaye *et al.*, 1983; Kappler *et al.*, 1983b; Meuer *et al.*, 1983c). Again, these are characteristics that would be expected of antibodies binding part of the T cell receptor. Third, we demonstrated that a mono-

clonal antibody, KJ1-26, raised against an ovalbumin-specific, IA^d-restricted T cell hybridoma, DO-11.10, failed to bind to subclones of this hybridoma which had lost the ability to bind to or respond to ovalbumin plus IA^d, again suggesting that the antibody concerned did indeed bind all or part of the receptor for ovalbumin plus IA^d (Haskins *et al.*, 1983). Fourth, this same clone-specific anti-receptor antibody, KJ1-26, was used to screen a large panel of independent T cell hybridomas prepared from T cells of BALB/c ($H\text{-}2^d$) mice primed with ovalbumin. The antibody bound to a single hybridoma of about 400 tested, and this T cell hybridoma turned out to have the same rare fine specificities, both for antigen and MHC, as the T cell hybrid. DO-11.10, against which the antibody was raised. This result could only be compatible with the reaction of the antibody with the antigen- and MHC-specific receptor on the T cell (Marrack *et al.*, 1983b).

Finally, all monoclonal antibodies so far described with properties of the type listed above precipitate similar surface molecules from target T cells. These molecules are disulfide-bonded heterodimeric glycoproteins with characteristics that are both reminiscent of yet distinguish them from immunoglobulins.

Although like antibodies these molecules are constructed of two different kinds of polypeptide chains, each molecule contains a single chain of each type. The two polypeptide chains are of similar molecular weights, being both about 43,000 in mouse (Allison *et al.*, 1982; Kappler *et al.*, 1983b). In man the more acidic, α, chain is between 45,000 and 50,000, and the more basic, β, chain is lower in molecular weight at about 40,000 (Meuer *et al.*, 1983a; Kappler *et al.*, 1983a). Both α and β chains are extensively glycosylated. In mouse both chains bear both N-linked and O-linked sugar residues. The polypeptide backbones, stripped by endoglycosidases or synthesized in the presence of tumicamycin, have been reported to be 31–32K (β chain) and 27–31K (α chain) (McIntyre and Allison, 1984; Kaye and Janeway, 1984). The molecular weights of the amino acid sequences predicted from cDNA clones suggest the lower of these molecular weights for the α chain is probably more accurate (Chien *et al.*, 1984a; Saito *et al.*, 1984a). The difference in molecular weights between human α and mouse β chains seems to be entirely due to more extensive glycosylation of the α chain in this species (Oettgen *et al.*, 1984). Probably because of different amino acid sequences in their variable regions, however, both chains vary in molecular weight and charge when isolated from different T cell clones (Kappler *et al.*, 1983b). In addition, peptide fingerprinting showed that α chains from different clones had peptides which distinguished them, and also peptides which they shared. This was also true for β chains, indicating that both chains had immunoglobulin-like variable and constant regions. Finally, peptide fingerprinting showed that α and β chains had no peptides in common, proving

that they were not derived from the same collection of genes (Acuto *et al.*, 1983; Kappler *et al.*, 1983a).

Apart from the disulfide bond between the two chains, data from experiments in which receptor polypeptides were reduced with limiting concentrations of 2-mercaptoethanol indicated that intrachain disulfide bonds also existed (Samelson and Schwartz, 1983). The interchain disulfide bond is the most sensitive to reduction (Samelson and Schwartz, 1983; J. Kappler, unpublished observations). Interestingly this parallels results with immunoglobulins in which the interheavy chain bonds are most easily destroyed by reducing agents.

Contrary to expectations the gross structural properties and peptide fingerprints of α and/or β chains could not be used to distinguish receptors on T cells specific for class I MHC products from those on class II specific cells. Class I- and class II-restricted cells all shared the same ^{125}I surface labeled peptides after digestion with trypsin or pepsin (Acuto *et al.*, 1983; Kappler *et al.*, 1983a). The possibility remained, however, that some part of the receptor buried deeply in the membrane, or in the cell cytoplasm, which would be inaccessible to the ^{125}I-labeling methods used in these experiments, might be different in cells with these different specificities. This idea has not been supported by molecular biological analyses (see below).

In summary the receptor on T cells for antigen plus MHC has been isolated using monoclonal antibodies. The molecule is a disulfide-bonded heterodimer of 85–90K. The two glycopolypeptide subunits appear unrelated to each other and to immunoglobulin. Each has variable and constant peptides. The overall structure of the molecule is therefore similar in design to the antibody molecule.

III. Properties of cDNA and Genomic Clones Encoding T Cell Receptor Proteins

Early in 1984 two groups reported the sequences of cDNA clones isolated from subtraction libraries of a human T cell leukemia (Yanagi *et al.*, 1984) or of mouse T cell hybridomas (Hedrick *et al.*, 1984a,b). The mRNAs which gave rise to these clones are expressed only in T cells, and the cDNA clones bind DNA which rearranges between the germ line and mature T cells. The amino acid sequences predicted by these clones are immunoglobulin-like, having two domains with properties very similar to those found in immunoglobulins, containing intrachain disulfide loops and other residues such as Trp-32, Gln-35, and Trp-178 at conserved positions. Each of these sequences also contains a leader sequence, a J-like region, a hinge-like portion concerning a cysteine residue, which is presumably involved in covalently linking the α and β chains, a transmembrane region and a short cytoplasmic tail (diagrammed in Fig. 1). Sites for N-linked glycosylation are also present.

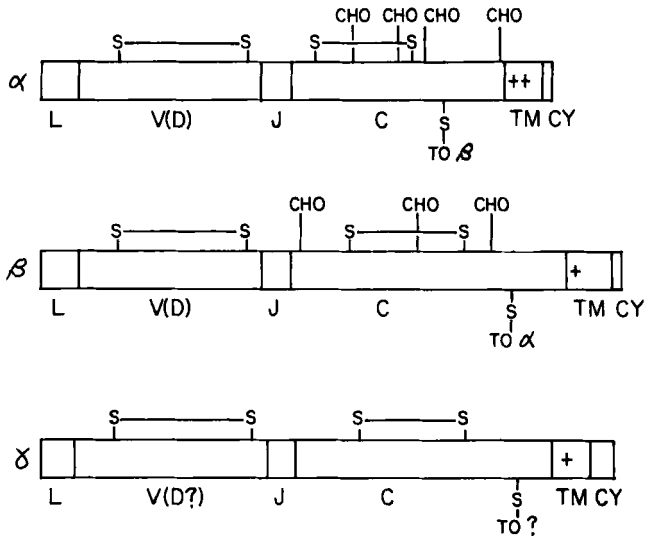

FIG. 1. Structure of mouse receptor and receptor-like polypeptides. The diagrams show to scale the predicted protein structures of mouse α, β, or γ polypeptides. The leader, variable plus D, J, constant domain, transmembrane, and cytoplasmic regions are indicated for each chain. Also shown are some of the cysteine residues, with their probable disulfide linkages. Sites for N-linked glycosylation (CHO) are shown for the constant regions only. Data are from Hedrick *et al.* (1984b), Saito *et al.* (1984a,b), and Chien *et al.* (1984a).

When the sequences of cDNA inserts isolated from different T cell hybridomas are compared it is clear that these genes are immunoglobulin-like in one other crucial respect since the predicted amino acid sequences of the first domain are clearly variable, whereas the second domain, hinge, transmembrane, and cytoplasmic region sequences appears (almost) constant. Extensive homology between the nucleotide and predicted amino acid sequences of the human and mouse cDNA clones shows that they code for the same chain of the T cell receptor. N terminal protein sequencing later showed that the chain encoded is that of the β chain (Acuto *et al.*, 1984; Hannum *et al.*, 1984a,b).

Many interesting conclusions have been drawn already from studies on these recently discovered β chain genes. First, the structure of these genes in the germ line has been analyzed (diagrammed in Fig. 2). The germ line structure of mouse and man is very similar. In each case there are two closely linked genes with very similar structures, exon sequences, and predicted amino acid sequences encoding two constant regions. Each constant region gene is constructed from four exons. The positions and lengths of the introns interrupting the two C region genes are almost identical. The exons

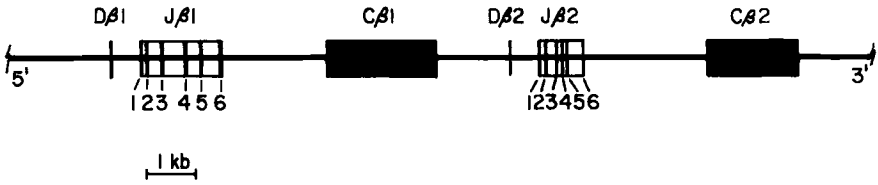

FIG. 2. Germ line structure of the mouse β chain locus. This is shown to scale without indication of the intron/exon structure of Cβ1 and Cβ2. Data are from Gascoigne *et al.* (1984), Malissen *et al.* (1984), Siu *et al.* (1984a,b), and Kavaler *et al.* (1984).

of each gene consist of one coding for the second, constant region, extracellular domain, a very small exon coding for the hinge-like region, one exon encoding the transmembrane region and finally an exon coding for the cytoplasmic tail and 3′ untranslated region (Gascoigne *et al.*, 1984; Malissen *et al.*, 1984). Most interestingly in mouse the coding regions of the two constant region genes differ by only 24 of their 318 nucleotides, whereas the 3′ untranslated regions of the two genes are different lengths, and also differ considerably in DNA sequence, being less than 50% homologous. The 24 base differences between the coding sequences for the two constant regions lead to only 4 amino acid changes, all in or near the transmembrane and cytoplasmic domains (Gascoigne *et al.*, 1984). The fact that the 3′ untranslated regions of these two genes are so dissimilar, coupled with the fact that the genomic organization of these genes in man is similar to that in mouse, suggests that duplication of the Cβ genes occurred before speciation of mouse and man. Gascoigne *et al.* (1984) have suggested a time about 120 million years ago. In that case, the conservation of nucleotide and coded amino acid sequences of the two constant regions is really remarkable, and implies strong, selective pressures on the sequences encoded, or perhaps very recent or very frequent gene conversions in the coding sequences. The idea that the amino acid sequence of these genes is strongly conserved by pressure for it to function in a particular way is supported by the fact that the translated amino acid sequences of human and mouse Cβ regions are also very similar, only 37 amino acids of 177 encoded by these four exons differ.

There is no evidence that one or other C region is selectively expressed by one type of T cell, since class I- and class II-restricted cells have been found expressing Cβ1, and likewise other cells of both specifities have been found expressing Cβ2 (Royer *et al.*, 1984; Kronenberg *et al.*, 1985; Hedrick *et al.*, 1985). The genomic structures of the two constant regions are similar in other ways. Both genes are preceded by a cluster of 6–7 functional J region genes, and about 500–600 bases upstream from each of these a D region segment is located (Gascoigne *et al.*, 1984; Malissen *et al.*, 1984; Siu *et al.*, 1984a,b; Kavaler *et al.*, 1984; Clark *et al.*, 1984). The approximate structure

of the entire locus is shown in Fig. 2. In functional T cells it has been shown that each D region can rearrange to any of the J regions in the adjacent cluster by a looping out-insertion mechanism. In addition Dβ1 can rearrange to any of the J region genes in the second cluster, again by looping out and insertion. In immature and mature T cells V region genes also rearrange to either D and/or J cluster. There is evidence that in some cases rearrangements may occur by mechanisms such as sister chromatid exchange or inversion (Chien *et al.*, 1984b; Siu *et al.*, 1984a,b; Kronenberg *et al.*, 1985; Born *et al.*, 1985).

In any case the mechanisms of rearrangement for these genes seem to be very similar to those used for immunoglobulin gene rearrangement in B cells. Thus D segments and the 5′ sides of J regions are flanked by heptamer/nonamer sequences similar to those which are used to indicate rearrangement sites for heavy and light chains (Siu *et al.*, 1984b; Kavaler *et al.*, 1984; Sakano *et al.*, 1979; Early *et al.*, 1980; Sakano *et al.*, 1980; Tonegawa, 1983). One difference was noted, however, in that if the rules worked out for immunoglobulin genes apply here, there is evidence that Vβ genes may have the option of rearranging directly to a J region gene. Thus D region genes are flanked by heptamer/nonamer sequences with spacers of 12 nucleotides on their 5′ sides, and 23 nucleotides on their 3′ sides. The heptamer/nonamer sequences of J region genes all have 12 base spacers on their 5′ sides. This implies that a V region gene with 3′ heptamer/nonamer sequences having a 23 base spacer could rearrange either to D or J (Siu *et al.*, 1984b; Kavaler *et al.*, 1984). Indeed there is some evidence from the sequencing of a β chain of a cDNA clone from the human T cell leukemia HPB-MLT that D region genes may sometimes be missing, though of course such sequences may theoretically be so small as to be invisible (Jones *et al.*, 1985).

Again as in the case of immunoglobulins there is evidence that non-germ line encoded nucleotides can be introduced at the point of V to D or D to J joining, leading to so-called N region diversity (Siu *et al.*, 1984b; Sakano *et al.*, 1981; Alt and Baltimore, 1982). All in all, it is clear that β chains use many of the strategies previously described for immunoglobulin genes to achieve diversity. This will be discussed at greater length below.

cDNA clones encoding α chain polypeptides have been isolated, like β chain cDNAs, from T cell-specific cDNA subtraction libraries (Chien *et al.*, 1984a; Saito *et al.*, 1984b) and also by the use of amino acid sequences of tryptic peptides from the α chain of the human leukemia, HPB-MLT (Sim *et al.*, 1984). In this latter case degenerate oligonucleotide probes were built to match the known amino acid sequences, and were used to isolate cDNA clones which encoded the α chains in question. Like β chains, both in mouse and man, α cDNA probes bind to mRNA expressed only in T cells and detect

genomic DNA which rearranges between the germ line and mature T cells. Again, there is extensive homology between the coding DNA sequences and translated amino acid sequences of mouse and man, though this is less marked than for β chains.

As for β chains, the amino acid sequences known or predicted for α chains suggest a strong relationship to the immunoglobulin family (Fig. 1). α chain polypeptides each have a leader sequence of about 20 amino acids, two immunoglobulin-like domains defined by intrachain disulfide bonds and other conserved residues, a J-like sequence, and hinge, transmembrane, and cytoplasmic regions (Chien *et al.*, 1984a; Saito *et al.*, 1984b; Sim *et al.*, 1984). There is evidence that D region sequences are optional, as for β chains (Sim *et al.*, 1984). The first immunoglobulin like domain varies in sequence from clone to clone as expected for a V region. The second, C region domain is somewhat surprising since it is unexpectedly short, the disulfide-bonded intrachain loop includes only 50 rather than the usual 70 amino acids, and lacks residues that are otherwise universally conserved in the immunoglobulin family such as Trp-148. On the other hand, the so-called hinge region, containing a cysteine residue presumably involved in cross-linking to the β chain, is more reminiscent of an immunoglobulin hinge than its counterpart in the β chain.

The supposed transmembrane region of human and mouse α chains contains 2 positively charged amino acids. This was surprising, since β chain transmembrane regions had previously been shown to contain one basic amino acid, and it had been suggested that α β dimers might be stabilized in the membrane by the presence of a complementary negatively charged amino acid in the α chain transmembrane region. The significance of the fact that the two chains possess jointly 3 basic amino acids in what is thought to be that portion of their sequences which spans the lipid bilayer of the cell will be discussed in greater detail below.

So far very little of the germ line structure of α chain genes has been reported. It is already clear, however, that, unlike Cβ, the locus contains only one functional C region gene, and there must be a very large intron between the Jαs and Cαs since no restriction enzyme used by us has shown rearrangements involving Jα on Southern blots using a Cα probe (J. Yagüe and W. Born, personal communication).

In summary cDNA clones encoding the α and β chains of the T cell receptor have now been isolated. They demonstrate that these genes are expressed exclusively in T cells and have properties very similar to those of immunoglobulins. The functional gene is composed of rearranged V, (D), and J segments and a constant region portion. The mechanisms causing rearrangement seem to be similar to those used by immunoglobulin genes in B cells.

IV. Other Receptor-Like Genes in T Cells

At least one other type of cDNA has been reported in T cells which has the characteristics of the receptor genes discussed above, but which does not appear to code for a protein of the T cell receptor as it is currently understood. While searching through a cDNA subtraction library made from a cytotoxic T cell clone, Saito *et al.* (1984a) discovered a cDNA clone which has a nucleotide sequence and predicted amino acid sequence very similar to those of α and β chains. The structure of the protein encoded by this cDNA clone, currently called the γ chain, is shown in Fig. 1. The molecule has leader, V,D,J,C, transmembrane, and cytoplasmic regions reminiscent in length and sequence to the β chain. The C region domain is the same length, for example, as the β chain and the transmembrane region contains, like the β chain, a single positively charged amino acid. Like its receptor counterparts γ chain genes are rearranged and transcribed only in T cells. Several properties, however, indicate that this chain is not part of the T cell receptor as we currently understand it. First, amino acid sequencing has not picked up in any receptor a sequence reminiscent of the γ chain. Second, this sequence has no Asn × Ser or Asn × Thr sequences, the sites used for N-linked glycosylation, and all mature receptor proteins so far examined have N-linked sugars (McIntyre and Allison, 1984; Kaye and Janeway, 1984). Third, the γ chain appears to have limited variability when the sequences of cDNAs isolated from different T cells are compared (Saito *et al.*, 1984a; Hayday *et al.*, 1985). Last, in the periphery, γ cDNA in a functional form appears to be expressed primarily or perhaps exclusively by cytotoxic T cells, though it does have a very interesting pattern of expression in the thymus (see below).

The significance of this intriguing cDNA clone has yet to be understood, though presumably much more will be known by the time this manuscript reaches print. Interest centers principally on whether the molecule has any role in development of the T cell repertoire in the thymus or is involved in the function of cytotoxic T cells, and whether a functional counterpart of the γ chain exists in helper T cells.

V. Rearrangement and Expression of T Cell Receptor Genes in the Thymus

Clearly, since T cells mature in the thymus and it is thought that the T cell repertoire for self-MHC is selected in this organ, there is much interest in the state of T cell receptor genes and proteins in the thymus (Zinkernagel *et al.*, 1978; Fink and Bevan, 1978). The expression of receptor genes can be examined at 3 different levels, by demonstration of DNA rearrangement, mRNA transcription, or protein expression on the cell surface. At the moment research is proceeding on all three subjects.

As far as DNA rearrangements are concerned it is clear from Southern blots that the first lymphoid cells to reach the thymus in ontogeny have no rearrangements of their β chain DNA (Born *et al.*, 1985; Snodgrass *et al.*, 1985a; Trowbridge *et al.*, 1985). α chain DNA rearrangements have yet to be examined. Measurement of band densities on Southern blots, and of rearrangements in thymocyte hybridomas has shown that β chain rearrangements begin at about day 14 of embryonic life in mouse thymuses. Hybridoma results show that D to J rearrangements precede by a day or two the rearrangement of V region genes, a sequence of events which is reminiscent of events at the heavy chain locus in B cells (Born *et al.*, 1985; Alt *et al.*, 1984). Evidence from hybridomas made from cells cultured in thymic rudiments shows that most of these rearrangements occur in the thymus, rather than by arrival in day 14 and older thymuses of bone marrow migrants which have rearranged their β chain genes elsewhere (W. Born, personal communication). Whether rearrangements can occur elsewhere in the animal has yet to be examined but might be expected to occur at a low rate, because alloantigen-specific T cells have been produced from nude mice (Gillis *et al.*, 1979). These T cells presumably bear T cell receptors (although their structure and production have not yet been studied). If the receptors on these cells are indeed similar to those on normal T cells, then rearrangements in these animals may be occurring at sites other than the thymus.

β chain DNA rearrangements in the thymus usually proceed so that eventually each cell contains rearrangements on both chromosomes, and even at both Jβ clusters on the same chromosome (Born *et al.*, 1985). Many of these rearrangements are partial, i.e., they involve D to J movements only without introduction of V region sequences. This is true in peripheral T cells too. Kronenberg *et al.* (1985), for example, report an I-A^k-specific helper T cell line produced by Dr. Delovitch which has 4 rearrangements in its β chain loci. Three of these are rearrangements of Dβ1 to the Jβ1 cluster or Dβ2 to the Jβ2 cluster with as upstream V regions and one involves a VDβ1Jβ1 combination. We have observed similarly complicated configurations in the β chain genes of normal partners in thymocyte hybridomas (Born *et al.*, 1985). These abnormal rearrangements are often transcribed (see below). Whether or not protein is produced from the transcripts, and whether or not this protein has any function has yet to be established.

As far as mRNA transcription is concerned, Northern blot analysis reveals that very little β chain mRNA is transcribed in day 15 mouse embryonic thymuses (Snodgrass *et al.*, 1985a,b). This increases by day 17 and plateaus in adult thymuses. α chain mRNA transcription lags by about 2 days. Surprisingly and interestingly, transcription of the mysterious γ chain is very high in embryonic thymus, peaking at day 15 of embryonic life and than falling off rapidly (Snodgrass *et al.*, 1985a; Raulet *et al.*, 1985). The frequency of different transcripts, estimated from frequencies in cDNA libraries from

different sources, is that α and γ chain comprise about 0.01% and β chain about 0.1% of the polyadenylated mRNA in day 17 thymuses whereas in adult mouse thymuses α chain mRNA is 0.01%, β chain mRNA 0.2%, and γ chain mRNA has dropped to 0.001% (Chien *et al.*, 1984a; Snodgrass *et al.*, 1985a).

The progression of events occurring in maturing embryonic thymocytes continues in adult cells. It has been shown that the least mature cells in adult mouse thymus have a surface phenotype which allows their isolation since they bear low amounts of Ly1 but no Ly2 or L3T4 (Mathieson and Fowlkes, 1984; Scollay *et al.*, 1984). Once isolated these cells have been shown to contain β chain and T3δ chain (see below) but not α chain mRNA, yet only some of the hybridomas made from these cells contain rearranged β chain genes (Samelson *et al.*, 1985b). The indications are, therefore, that thymocyte maturation proceeds in the adult as it does in the embryo by sequential rearrangement and expression within the thymus of β and then α chain genes.

The molecular weight of α, β, and γ chain mRNA and sequencing of cDNA clones all indicate that up to 50% of their mRNA in thymus may be transcribed from genes having D to J rearrangements without upstream V regions. These transcripts are 0.2–0.3 kb shorter than those of fully rearranged complexes (Yanagi *et al.*, 1984; Clark *et al.*, 1984; Siu *et al.*, 1984b; Chien *et al.*, 1984; Snodgrass *et al.*, 1985a,b; Raulet *et al.*, 1985). Whether or not these are translated into protein, and whether or not this protein is of any functional significance has yet to be determined. Interestingly, similar transcripts have been described in B cells (Reth and Alt, 1984).

The production of cell surface receptor protein in thymus has also been determined, in several ways. The most satisfactory method would involve a monoclonal antibody which would react with all receptors of a particular animal, and which could therefore be used to precipitate all receptors, or stain all receptor-bearing cells. Unfortunately such a monoclonal antibody does not yet exist, so other approaches have been taken.

We have used a monoclonal antibody we have recently produced, KJ16-133, which recognizes an allelic determinant on the β chain of about 20% of T cells in most mouse strains (Haskins *et al.*, 1983). The nature of the determinant recognized by this antibody is not known at the moment, though we think it may react with a particular D or J sequence. In any event, this antibody does not react with thymocytes until about day 17 of mouse embryonic life. At this point it stains a very low percentage of cells very faintly. The percentage of cells stained and intensity of staining gradually increase as the thymus matures until, in the newborn thymus, the antibody stains about 10% of cells with a spectrum of intensities ranging from practically none, to levels about one-fifth those of mature T cells. This pattern is similar to that observed

for immature cells in adult thymus. Mature, cortisone-resistant, peanut agglutinin-positive thymocytes stain as strongly as peripheral T cells (Roehm *et al.*, 1984a).

These results suggest that receptor proteins are not expressed on the surface of thymocytes until day 17 of mouse fetal life, and that some immature thymocytes, even in adults, bear no receptors at all. At no point do immature thymocytes bear more receptors/cell than peripheral T cells, and usually they bear considerably less.

Similar conclusions have been drawn from other studies, including those of human thymocytes in which the presence of receptors has been correlated with the T3 protein complex (see below). Receptors on mouse and human thymocytes have also been picked up using the so-called off-the-diagonal method originally used in this context by Goding and Harris (1981; Acuto *et al.*, 1984; Snodgrass *et al.*, 1985b).

Overall, the DNA, RNA, and protein experiments agree very well with each other, and suggest a scheme in which the first lymphoid cells to enter the thymus have no rearranged receptor genes and express no receptor mRNA or protein. Early thymocytes rearrange and express only γ chain genes, with products of currently unknown function. A day or two later β chain genes begin rearrangement and several days later fully VDJ rearranged β chain genes are formed and transcribed. Expression of α chain mRNA lags a day or two behind this, and its appearance correlates with the first appearance of receptor proteins on the surface of these cells.

VI. Expression of Receptor Genes and Proteins on Peripheral T Cells

The rearrangement and expression of T cell receptor genes in peripheral T cells has been examined in some depth. Not unexpectedly, given the data observed in the thymus, β chain genes are extensively rearranged in peripheral T cells, and a single cell may have multiple rearrangements (Kronenberg *et al.*, 1985; Hedrick *et al.*, 1985). The rearrangements of α chain genes in peripheral T cells has not yet been examined in much depth, γ chain genes seem to be almost universally rearranged.

Interpretation of Southern blots and sequencing of cDNA clones have shown that all functional peripheral helper or cytotoxic T cells or T cell hybridomas so far examined express mRNA for at least one complete β chain protein. This is not true for suppressor T cells, for which contradictory results have been obtained. In the mouse, a number of suppressor T cell hybridomas defined by their ability to secrete antigen-binding suppressor factors have been shown to have completely lost all β chain genes derived from the normal T cell parent (Kronenberg *et al.*, 1985; Hedrick *et al.*, 1985). It is not yet known how this has happened, though probable chromo-

some loss is involved. In man suppressor T cells, usually defined less stringently by their ability to inhibit pokeweed mitogen induction of immunoglobulin secretion by B cells, have rearranged β chain genes (Toyonaga *et al.*, 1984; Royer *et al.*, 1984). Overall, the interpretation of these experiments is that the β chain of the T cell receptor is probably not involved in the formation of antigen-specific suppressor factors, unless all the mouse suppressor T cell hybridomas so far tested use a β chain encoded by the tumor cell parent of the hybridomas (BW5147) to form these secreted products. Relevant to this argument is the fact that BW5147 does encode a complete β chain which can be found in surface receptors on T cell hybridomas under appropriate conditions (Yagüe *et al.*, 1985; R. Kubo, personal communication).

At the time of writing not much is known about the rearrangement and expression of α chain genes in peripheral T cells.

Since the β chain locus contains two different Cβ genes, linked to two different D regions and J clusters, it is sensible to ask whether these are differentially used by, for example, class I- or class II-restricted T cells. So far, as noted above, there is no evidence for this (Royer *et al.*, 1984; Kronenberg *et al.*, 1985; Hedrick *et al.*, 1985). Both J region clusters and Cβ genes can be used by T cells with either specificity. Indeed, the recent finding that the same V region gene can be used by a T cell specific for sheep red blood cells plus I-A^b or by a D^d-specific T cell line suggests that no part of the β chain defines irrevocably even the class of MHC specificity displayed by the T cell expressing it (Hedrick *et al.*, 1985). Whether or not this will also be true of the α chain remains to be seen. So far, in fact, it is only γ chain mRNA which may encode protein in peripheral cytotoxic T cells only, which distinguishes at the level of receptor or receptor-like genes class I- from class II-restricted T cells (Saito *et al.*, 1984a; Hayday *et al.*, 1985).

The levels of expression of different receptor mRNAs have been compared in peripheral T cells, as they have in thymocytes, by comparing the frequencies of the different genes in peripheral T cell cDNA libraries. β chain expression is most frequent, at 0.1% of total mRNA, followed by α chain mRNA, at 0.03% and γ chain mRNA, at 0.001% (Chien *et al.*, 1984a).

The presence of receptor protein on peripheral T cells has been tested by several means. Using the anti-allotype antibody, KJ16-133, we have shown that this antibody reacts with about 20% of our T cell hybridomas which bear antigen-specific, MHC-restricted receptors (Haskins *et al.*, 1983). This percentage compares well with the numbers of peripheral T cells in most mouse strains with which this antibody reacts, about 20%. Taken together these data suggest that in mice the vast majority of peripheral T cells do indeed bear receptors of the type discussed in this article. This idea is bourne out by the fact that in man all peripheral T cells bear T3, the protein complex

known to be tightly associated with the receptor on the T cell surface (Reinherz *et al.*, 1979, see below).

In summary, therefore most, if not all, peripheral T cells seem to express at least one functional β chain gene and, in fact, intact receptor protein on their surfaces. So far, no features of the receptor, either at the DNA or protein level (discussed in a previous section), appear to distinguish those restricted by class I or class II MHC products. This is surprising in view of the fact that within MHC-class cross-reactions for T cells have frequently been reported, but no T cell has yet been described which cross-reacts between class I and class II indicating that something must distinguish the two types of receptors.

VII. V Region Repertoire and Variability

Several attempts have been made to estimate the extent of variability of β chain V region genes. At the time of writing it is known that on the whole Vβs belong to small families. Thus, if a particular Vβ gene is used to probe Southern blots of germ line DNA only a few bands, sometimes only one, hybridize strongly. Immunoglobulin V regions do not behave in this way, and under relatively stringent conditions may bind to more than 20 bands in Southern blots. Not only do individual V genes bind to small families, but the corollary of this is also true, i.e., different Vβs are quite unlike each other both in nucleotide and predicted amino acid sequence. Patten *et al.* (1984) have compared the predicted amino acid sequences of 7 Vβ genes on a variability plot, and report extensive variability throughout the molecule, not only in positions analogous to hypervariable regions in immunoglobulins. Based on this they suggested that the binding site(s) of the T cell receptor to antigen plus MHC may not be limited to the cleft defined by complimentarity determining regions in antibodies.

Even more remarkably, Patten *et al.* (1984) have shown that particular Vβ genes are expressed by T cells at unexpectedly high frequencies. By probing thymocyte and spleen cDNA libraries with Cβ and individual Vβ probes they estimated that up to 38.9% of the Vβ mRNA could be accounted for by only 4 different sequences. On the other hand, another Vβ did not hybridize to any genes in the library, suggesting that it was used very rarely. Even this may not be the case, however, since this same Vβ gene has been found used by two T cell hybridomas specific for cytochrome *c*/I-E^k and another specific for hen egg lysozyme/I-A^b (Goverman *et al.*, 1985). Barth and Hood (personal communication) have continued this analysis and estimate that less than 25 Vβ genes exist in the mouse germ line repertoire. Their arguments are based on the assumption that all Vβ genes are expressed with equal frequency, which is probably not true, so this may be an underestimate.

On several occasions Vβ cDNA sequences have been compared with those of their germ line counterparts, and the conclusion drawn that somatic variation is relatively limited. In fact frequently the cDNA sequences of antigen-stimulated peripheral T cells are identical to those of the germ line gene (Chien *et al.*, 1985). The implication of this is that variation of at least Vβ genes is not essential in order for the cell to be selected in and migrate from the thymus. Nor does it necessarily occur during responses to antigen *in vivo*. This does not mean that somatic variation may not occur, it simply implies that it is not essential.

Somatic variation has been demonstrated in the receptor genes of a T cell hybridoma grown *in vitro* (Augustin and Sim, 1984). Peptide maps of the receptor suggested that in different variants both Vα and Vβ genes varied. Of particular interest is the fact that variation in receptor V genes was accompanied by changes in the specificity of the T cell hybridoma for class II molecules, suggesting that these variations may be used to identify contact points between the receptor and its targets.

The different specificities of T cells for their targets will presumably be attributable to different combinations of Vβ, Dβ, and Jβ on which N region diversity and somatic variation are imposed, to yield finally a very large number of receptor sequences. It is possible that there are relatively few Vβ genes, but this may be compensated for by a larger number of Jβ possibilities than is usual for immunoglobulin genes. All in all it is likely that the T cell repertoire is very large, perhaps as large as that of antibody molecules.

VIII. Chromosomal Mapping of T Cell Receptor Genes

Once cDNA probes for these genes were available, the chromosomal positions of the genes were assigned by Southern blot analysis of human hamster or mouse hamster somatic cell hybrid line DNA. In some cases use of cells with particular chromosomal breaks allowed the position of the gene on the chromosome to be assigned.

In mouse, the location of β chain genes was also mapped after the discovery of restriction enzyme length polymorphisms in different strains. For example several restriction enzymes show restriction enzyme length polymorphisms in β chain genes comparing SJL mice with most other strains. When these polymorphisms were assigned to a collection of BALB/c × SJL recombinant inbred strains it was found that they mapped close to the κ chain locus on chromosome 6, and that these polymorphisms were distributed in these particular recombinant inbred strains concordantly with the reactivity of T cells in these strains with the anti-T cell receptor allotype antibody, KJ16-133. Examination of the distribution of the restriction enzyme length polymorphisms with KJ16-133 reactivity and reaction with anti-

TABLE I
CHROMOSOMAL LOCATION OF T CELL RECEPTOR AND RECEPTOR-LIKE GENES

Gene	Chromosomal location	References
Mouse α	14 D1-D2	Kranz *et al.* (1985)
Mouse β	9.6 cm proximal to K/Ly2 on chromosome 6	Lee *et al.* (1984); Caccia *et al.* (1984); Epstein *et al.* (1985); Roehm *et al.* (1985)
Mouse γ	13 A2-A3	Kranz *et al.* (1985)
Human α	14q11	Croce *et al.* (1985); Jones *et al.* (1985)
Human β	7q32	Caccia *et al.* (1984); Morton *et al.* (1985)
Human γ	7p15	Murie *et al.* (1985)

Lyt2.2 antibody in (CBA/J × SJL)F_2 animals has allowed a rather precise mapping of β chain genes in mice.

The results of all mapping studies so far reported are listed in Table I.

There is some interest in the idea that chromosome breaks characteristic of particular human diseases may map in positions near receptor genes. In this regard it is interesting that the positions of the α, β, and γ genes have already been identified as frequent sites of breakages in the peripheral blood T cells of individuals with ataxia telangectiasia (summarized by Fiorilli *et al.*, 1985) and also 14q11 has been identified as a break point in several human tumors (Hecht *et al.*, 1984).

Finally, two of these genes are located on chromosomes already known to bear immunoglobulin genes, κ on chromosome 6 in mice and heavy chains on chromosome 14 in man and two of these genes are on the same chromosome, i.e., α and γ on human chromosome 7. Although α chain genes are apparently more than 40 cM from heavy chain genes on human chromosome 14, however it is possible that these associations are not coincidental and represent present day indications of ancient duplications.

IX. Allelic Exclusion of T Cell Receptor Genes

It is already clear from the study of β chain genes that rearrangement at one locus in the cell does not preclude rearrangement of other β chain genes (Kronenberg *et al.*, 1985; Hedrick *et al.*, 1985; Born *et al.*, 1985), though it remains to be seen whether these events give rise to the expression of more than one functional protein in the same cell. There is evidence at the protein level, however, that usually only one allele is expressed in any given cell. This was established using the anti-β chain allotype antibody, KJ16. For example, in CBA/J mice this antibody binds about 16% of T cells, and in SJL animals, none. Only about 9% of (CBA/J × SJL)F_1 T cells bind the antibody, but those that do react, bind the same amount as positive cells in the CBA/J

parent (Kappler *et al.*, 1984; Roehm *et al.*, 1985). These results are consistent with allelic exclusion, at least at the level of protein synthesis, of β chain genes in most T cells. Whether or not this occurs, as is now thought for B cells, by suppression of further rearrangements once a functional receptor protein or polypeptide has been expressed, or whether the chances of more than one successful rearrangement in the same cell are simply too low to allow frequent expression of more than one β chain/cell has not yet been established.

X. Association of the Receptor and T3

Some years ago a very interesting antibody identified a collection of proteins on human T cell surfaces now called T3 (Reinherz *et al.*, 1979; Ledbetter *et al.*, 1981; Meuer *et al.*, 1984). This complex consists of three polypeptides two of which are glycosylated [T3 γ(25K) and T3 δ (20K)] and one of which is not [T3ε (20K)] and, because it is difficult to label on intact cells with aqueous reagents and ^{125}I, it is thought to be extensively buried in the cell membrane (Borst *et al.*, 1983, 1984; Oettgen *et al.*, 1984). Anti-T3 antibodies also sometimes coprecipitate receptor molecules from T cells, suggesting that these are closely associated with the T3 complex (Meuer *et al.*, 1984; Oettgen *et al.*, 1984). Other data support this idea. For example, Meuer *et al.* (1984a) have shown that the receptor and T3 cocap on T cell surfaces. Like anti-receptor antibodies, anti-T3 antibodies may stimulate T cells to divide and secrete interleukin 2 or alternatively block responses to antigen (Reinherz *et al.*, 1980; van Wauwe *et al.*, 1980; Chang *et al.*, 1981; Landegren *et al.*, 1982). Variants of the human T cell leukemia, JURKAT, have been used to show that T3 is probably not expressed on T cell surfaces in the absence of the receptor, and vice versa (Weiss and Stobo, 1984). Finally cross-linking experiments have shown that the γ chain of T3 is closely associated with the receptor in the membrane of human T cells (Brenner *et al.*, 1985).

T3 is thought to be involved, not only in the association of the T cell receptor with the T cell plasma membrane, but also with the transmission of signals delivered by binding of ligands to the receptor to the interior of the cell. Anti-T3 and anti-receptor antibodies both induce depolarization and Ca^{2+} fluxes in T cells for example (O'Flynn *et al.*, 1984, 1985; A. Weiss *et al.*, 1984; M. J. Weiss *et al.*, 1984; Oettgen *et al.*, 1985), and in mice T cell activation via the receptor causes phosphorylation of a protein apparently analogous to the human T3 δ chain (Samelson *et al.*, 1985a).

T3 has yet to be completely characterized in mice. cDNA clones encoding the T3 δ chain have been isolated in man (van den Elsen *et al.*, 1984), and analogous clones in mice have now been isolated, (van den Elsen *et al.*,

1985). Cross-linking experiments have shown that the receptor is associated with a T3-like molecule in the membranes of mouse T cells, but the definitive antibody has yet to be isolated (Allison and Lanier, 1985).

XI. Is the Receptor Responsible for Specific Binding to Both Antigen and MHC by T Cells?

A long standing and not yet resolved debate has been conducted over the way in which T cells simultaneously recognize antigen and products of the MHC. Originally, two theories were suggested: that T cells use two different receptors to recognize antigen and the MHC product, or that they use a single receptor to recognize a combination of the two (altered self hypothesis) (Janeway *et al.*, 1976; Zinkernagel and Doherty, 1977; Zinkernagel *et al.*, 1978; von Boehmer *et al.*, 1978; Langman, 1978; Blanden and Ada, 1978; Cohn and Epstein, 1978; Rosenthal, 1978). The solution to this problem has yet to be undisputably worked out, however, recent experiments have favored some variation of the latter idea. Although this subject is really a subject for a full review article of its own, it might be useful at this point to summarize the data which support the latter theory and also discuss the experiments which suggest that the receptor, as described in this article, is indeed the molecule on T cell surfaces responsible for specific recognition of both antigen and MHC.

Ultimately, of course, the experiment required is a demonstration that isolated receptor proteins bind to the expected complex of antigen plus MHC. Experiments with isolated, univalent, detergent-treated receptors have so far failed to illustrate this (personal observation), not surprisingly perhaps given some of the points raised below. In lieu of this a number of experiments have shown that antigen and MHC are probably bound by a single receptor.

These include the observation that T cells do not *bind* to target cells unless these cells bear both the correct antigen and MHC product (Ben-Sasson *et al.*, 1975; Swierkosz *et al.*, 1978; Ziegler and Unanue, 1979). Dual recognition cannot therefore be attributed to a requirement for engagement of receptors for antigen, and receptors for MHC for cell activation only. This explanation is also hard to apply to T cells specific for MHC products in the absence of antigen. A second experiment which supported, in a completely different way, the idea that T cells have only a simple receptor was done as follows. Some years ago we produced a T cell hybridoma which bore recepetors inherited from 2 different T cell parents, for chicken ovalbumin plus I-A^k, and for keyhole limpet hemocyanin plus I-A^f. We reasoned that if two different receptors were really used for antigen recognition and MHC binding, the hybrid cell should respond with the specificities of its parents,

as well as the mixed specificities, for chicken ovalbumin plus I-A^f or for keyhole limpet hemocyanin plus I-A^k. In fact the hybrid cells responded only with parental specificities, suggesting that independent receptors were not used to respond to antigen and to MHC (Kappler *et al.*, 1981).

In two other series of experiments Hunig and Bevan (1982), Heber-Katz *et al.* (1982), Hedrick *et al.* (1982) and Hansburg *et al.* (1983) supported the same idea. Hunig and Bevan (1982), for example, showed that the specificity of T cells for antigen was affected by the MHC product being recognized. Thus, a particular cytotoxic T cell clone was able to recognize C3H or DBA/2 minor antigens in association with K^k only, but BALB/c minor antigens in association with K^k or D^d. This was interpreted as proving that different antigens interact with different H-2 antigens in different ways, and T cell receptors may thus recognize different combinations in the complexes, a prediction of an altered-self hypothesis.

These experiments all illustrated the idea that a single receptor molecule on T cells recognizes both antigen and MHC, but they did not prove that the receptor molecule concerned was that discussed earlier in this article. This was established by a number of experiments (mentioned above) including the fact that antibodies against the receptor inhibit specifically response, and binding to antigen plus MHC by the T cell bearing it. Even more strikingly, these antibodies when rendered polyvalent or sometimes in soluble form, stimulate target T cells (Kaye *et al.*, 1983; Kappler *et al.*, 1983b; Meuer *et al.*, 1983c). This is true even if the target cell be specific for antigen plus an MHC product, showing that the antibody can substitute for both stimuli (Kaye *et al.*, 1983; Kappler *et al.*, 1983b).

In an attempt to show that the molecule recognized by these antibodies was indeed responsible for the entire specificity of T cells we used a clone-specific anti-receptor antibody, KJ1-26, raised against the receptor on a chicken ovalbumin plus I-A^d-specific T cell hybridoma, DO-11.10, to screen T cell hybridomas. As described earlier second, and later third independent T cell hybridomas were found which bound the antibody (Marrack *et al.*, 1983b; Yagüe *et al.*, 1985). The molecule precipitated from the cells had identical peptide fingerprints when isolated from different cell lines (Kappler *et al.*, 1983a). Even more strikingly the specificities of these three hybridomas, both for antigen and MHC were identical, i.e., all recognized the same tryptic peptide of ovalbumin in association with either I-A^d or I-A^b. This is a very rare specificity among ovalbumin-specific BALB/c T cells (1/150, approximately). Overall these results proved that the molecule recognized by this antibody was responsible for recognition of both antigen and MHC. This does not imply necessarily that other molecules in T cell surfaces are not responsible for non-clone-specific binding to determinants on target cells (see below).

Other experiments have supported the idea that α and β chains only contribute to T cell specificity. For example we have produced variants of the T cell hybridoma, DO-11.10 which lack either the hybridoma-specific α or β chain. These variants have lost the ability to recognize ovalbumin plus I-A^d. Fusion of an α^- to a β^- hybridoma restored receptor function (Yagüe *et al.*, 1985). While not conclusive, these experiments again suggest that T cell specificity, at least in class II-specific T cells, can be accounted for entirely by receptor α and β chains. The existence of γ chain mRNA in cytotoxic T cell lines still leaves open the possibility that some other surface molecule may play the same as yet unsuspected role (see below).

XII. Interactions of T Cells with Their Targets

Clearly the receptor as it has been discussed in this article is involved in the binding of T cells to their cellular targets. The experiments described in the previous section also imply that this receptor is responsible for the recognition of both antigen, and a polymorphic component of the MHC by T cells. There is much evidence, however, that other T cell surface molecules are also involved in the interaction of T cells and their targets.

Most of these molecules were identified in the late 1970s and earlier in this decade by monoclonal antibodies produced from animals immunized with pooled T cells. The antibodies, and some of the names and characteristics of the proteins to which they bind, are listed in Table II. Even though these proteins are really a subject for an entire review, some of their properties are relevant to the subject of the T cell receptor and will be discussed here.

TABLE II
T CELL SURFACE MOLECULES WITH POSSIBLE ROLES IN TARGET RECOGNITION

Molecule		Approximate molecular weight(s) ($\times 10^{-3}$)	Reference
Mouse	Man		
LFA-1	LFA-1	180,95	Kurzinger *et al.* (1981); Pierres *et al.* (1982)
	Leu5, T11, CD2	55	Howard *et al.* (1981); Kamoun *et al.* (1981); Bernard *et al.* (1982)
Lyl	Leu1, T1, CD5	69	Ledbetter *et al.* (1981)
Ly2	Leu2a, T8, CD8	35,32,30	Ledbetter *et al.* (1981)
L3T4	Leu3, T4, CD4	62	Ledbetter *et al.* (1981); Dialynas *et al.* (1983); Terhorst *et al.* (1980)
T3	Leu4, T3, CD3	25,25,20	Ledbetter *et al.* (1981)
Thy 1		25	Trowbridge *et al.* (1978)

Some of these molecules are found on all, or almost all, peripheral T cells in man and/or mouse. These include lymphocyte functional antigen-1 (LFA-1), T11, T3, and Thy 1 (in mouse). Other molecules are found on subpopulations of peripheral T cells, these include T8 in man or Ly2 in mouse, and Leu 3, T4 in man or L3T4 in mouse. It is well known that T8/Ly2 is chiefly expressed by class I-reactive and L3T4 by class II-reactive T cells (Cantor and Boyse, 1975; Swain, 1981; Krensky *et al.*, 1982; Spits *et al.*, 1982; Meuer *et al.*, 1982b).

The idea that some of these molecules are involved in the recognition of target cells by T cells derives from the fact that many of these antibodies inhibit responses of pooled T cells. For example, both anti-leukocyte functional antigen-1 (LFA-1) and anti-L3T4 block mixed lymphocyte reactions (Davingnon *et al.*, 1981; Pierres *et al.*, 1982; Engleman *et al.*, 1981; Biddison *et al.*, 1982; Meuer *et al.*, 1982a). Anti-LFA-1 and anti-Lyt2 block cytotoxic T cells bearing these molecules (Davingnon *et al.*, 1981; Pierres *et al.*, 1982; Nakayama *et al.*, 1979; MacDonald *et al.*, 1982; Meuer *et al.*, 1982a). On the other hand, other molecules have been thought interesting because antibodies against them, particularly in polyvalent form, stimulate target T cells. This is true for antibodies to T1 or Ly1, T3, T11, and Thy 1 (Meuer *et al.*, 1983c, 1984; Fox *et al.*, 1985; Shimizu *et al.*, 1982).

Clearly the fact that so many molecules have been identified, none of which is the clonally distributed specific antigen receptor itself, but all of which seem to have some effect on the recognition of antigen by T cells, indicates that these molecules will not all have the same function. T3 seems to operate as part of the T cell receptor complex on the surface of the cell (see above), but there is no evidence that the other molecules listed in Table II are associated with the receptor at all. What could their roles therefore be?

Hypotheses vary depending on the molecule under study. It has been suggested, for example, that T11 plays some role in a route of T cell stimulation that does not involve the antigen/MHC receptor, and that the balance of receptor and T11 mediated signals in thymocytes causes tolerance induction in these cells (Fox *et al.*, 1985; E. Reinherz, personal communication). An entirely different idea has been suggested for Ly2 and L3T4. Antibodies to these proteins seem to affect different T cell clones differently, and by and large T cells clones which appear by other criteria to be the most sensitive to antigen are those which are least easily inhibited by anti-Ly2 or anti-L3T4. It has therefore been suggested that Ly2 and L3T4 act by binding to ligands on target cells and, thus, increase the overall avidity of T cells for their targets. The ligands recognized have been postulated to be nonpolymorphic regions of class I or class II molecules (MacDonald *et al.*, 1982; Marrack *et al.*, 1983a; Greenstein *et al.*, 1984; Watts *et al.*, 1984). Indeed there is some evidence to support this notion. For example, the response of a T cell hybridoma specific

for the class I antigen, D^d, but bearing uncharacteristically L3T4 instead of Ly2, is inhibited by anti-L3T4 only if the target cell involved is class II^+ (Greenstein *et al.*, 1984). Even more strikingly a T cell hybridoma reacting with antigen and/Ia on an artificial planer membrane containing no other proteins is inhibited by anti-L3T4 antibodies (Watts *et al.*, 1984; D. Gay, personal communication).

It is, of course, still possible that anti-Ly2 or anti-L3T4 act by delivering some sort of inhibitory signal to T cells that cannot be overcome by the recognition of antigen plus MHC by the receptor. This seems unlikely, however, because both anti-L3T4 and anti-Ly2 interfere with T cells binding to their targets. This does not seem to be due to an effect on receptor distribution or function because anti-L3T4, at least, does not interfere with the binding of or delivery of stimulatory signals by anti-receptor antibodies (personal observation). Some contradictory evidence still exists, however, and the matter has yet to be completely resolved.

The role of LFA-1 is even less well understood. This antigen has analogs on cell types other than T cells (Trowbridge and Ovary, 1981). On macrophages, for example, the related molecule, Mac 1) (Springer *et al.*, 1979) is thought to be the receptor for C3bi (Sanchez-Madrid *et al.*, 1983). Anti-LFA-1 antibodies are inhibitory for most, but not all, T cell clones, but surprisingly do not seem to affect the responses of the same T cell to all targets (Golde *et al.*, 1985). Whether this means that LFA-1 acts by binding to ligands which are differentially expressed by different targets, in a fashion analogous to L3T4 and Ly2, has yet to be resolved.

XIII. Concluding Remarks

Overall, however, it seems that T cell binding to and recognition of its target may involve not only the receptor, and its antigen plus MHC ligand, but also other T cell proteins and ligands on the target cell surface. This need for accessory molecules may reflect the relatively low affinity of the T cell receptor for antigen plus MHC (as exemplified by our inability to demonstrate that the isolated receptor binds to its expected target), and may also reflect the fact that the receptor, antigen, and MHC molecule must form a trimolecular complex before the interaction is stabilized. It appears that, except in rare cases, the T cell receptor does not have detectable affinity for either antigen or MHC alone, likewise, it is usually not possible to demonstrate any affinity of the antigen for MHC (Shimonkevitz *et al.*, 1984), although there are exceptions to all these statements (Siliciano *et al.*, 1980; Glimcher and Shevach, 1982; Werdelin, 1982; Endres *et al.*, 1983; Rock and Benacerraf, 1983; Rao *et al.*, 1984). It is therefore our belief that T cells engage their targets transiently using low affinity reactions between, for

example, L3T4 molecules and their ligands. During this short binding period receptors diffuse on the T cell surface, and antigen or antigen fragments and MHC molecules diffuse on the surface of the target cell, and trimolecular complexes of these components form and stabilize the interaction between the two cells such that signals can be transmitted, either to the T cell, or to its target or both.

This may seem like a very involved method for the recognition of a ligand on one cell by a receptor or another, but presumably the system is subject to constraints as follows. First, T cells which bind free antigen would not be efficiently triggered, because stimulation of virgin T cells requires not only interaction with their receptors, but also accessory cell-derived lymphokines such as interleukin-1, thus only T cells which bind antigen on accessory cells are triggered in a primary response. Moreover, T cells which bind free antigen would also be useless at the effector cell level, since T cells are designed to interact with other cells. Last, T cells should not bind so effectively to their targets that they cannot release them and move on to act on another target cell. Release of target cells could be achieved either by capping and regenerating the receptor or by designing the entire interaction so that it occurs under low affinity, reversible conditions. Perhaps both mechanisms operate.

Thus, it is clear that the discovery of T cell receptor proteins and genes has solved the initial problem in understanding how T cells interact with their targets. Many questions remain unresolved at the time of writing, however, though the tools to their solution are now in our hands. Unanswered questions include that of the nature of the complex T cell receptors from with antigen and MHC, mechanisms for thymic restriction, and the means by which T cells interact with their targets.

ACKNOWLEDGMENTS

The authors would like to thank Kelly Bakke and Donna Thompson very much for their secretarial help. This work was supported by U.S. Public Health service Grants A1-18785 and 17134 and ACS research Grants IM-49 and 319, and during the tenure by John Kappler of an American Cancer Society Faculty Research Award, 218.

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ADVANCES IN IMMUNOLOGY, VOL. 38

Immune Response (*Ir*) Genes of the Murine Major Histocompatibility Complex

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I. Introduction

The origins of the concept of the genetic control of the immune response can be traced back to the medical literature of the late nineteenth and early

twentieth century (see Longo *et al.*, 1981, for a review). As early as 1877 German physicians had noted that susceptibility to diphtheria was prevalent among certain family groups and had postulated that there might be a genetic basis for this observation (Jacobi, 1877). However, the distinction between genetic and environmental factors was not clearly discerned until the phenomenology was transferred into the laboratory for careful study in animal models. In 1943 Fjord-Scheibel reported that the magnitude of antibody production in guinea pigs in response to immunization with diphtheria toxin varied widely among individual animals. She then selectively mated high responder animals to each other and low responder animals to each other for 6 generations and studied the genetic inheritance of responsiveness at each generation. The experiment revealed that both high and low responsiveness were genetically inherited, although high responsiveness was easily inbred (first generation), whereas low responsiveness required many more generations (five) to establish. These experiments conclusively demonstrated that immune responsiveness was controlled by genetic factors and led Scheibel to suggest that high responsiveness was the dominant trait. However, as pointed out by Carlinfanti (1948), these data were also compatible with the notion that responsiveness was recessive, if the high-titered guinea pigs chosen for mating were already homozygous.

Throughout the 1950s this seminal observation was repeatedly confirmed using a variety of protein and cellular antigens and in a variety of animal models, in particular with inbred strains of mice (see Longo *et al.*, 1981, for a review). In a few cases, attempts were made to determine the number of genetic factors involved using F_2 and backcross analyses. However, the quantitative data obtained were largely overlapping among groups and required elaborate statistical analysis, involving many assumptions about the population, in order to reach any conclusions (Stern *et al.*, 1956). As a consequence, the importance of these observations did not really become clear until the early 1960s when immunologists began to examine the immunogenicity of synthetic polypeptides and small proteins, such as insulin. These molecules greatly facilitated the genetic analysis, because they presented the immune system with a limited degree of antigenic complexity and thus allowed a clear-cut distinction between responder and nonresponder animals. This, in turn, facilitated the discovery of two key facts about the genetic control of the immune response. One was that the phenomenon appeared to be controlled by a single autosomal dominant genetic locus. The other was that this regulation appeared to be antigen specific. It was to the great credit of the early workers in this field, in particular Benacerraf, McDevitt, and Sela, that they realized the importance of these two observations and chose to devote most of their research careers to the study of *Ir* genes.

In this review we will begin with a detailed analysis of the early experiments of these scientists, culminating in the discovery by McDevitt that these genes were linked to the major histocompatibility complex (MHC). From that point we will follow one of the major unifying threads in cellular immunology which ended in the identification of MHC-encoded molecules as the *Ir* gene products and the merging of the two phenomena: *Ir* gene control and MHC restriction (the requirement for T cells to recognize an MHC-encoded molecule in addition to the antigen in order to become activated). For a comprehensive review of the phenomenon of MHC restriction see R. Schwartz (1984). Finally, we will outline the current thinking on the mechanism(s) by which MHC-encoded molecules influence the T cell repertoire in an antigen-specific manner.

The topic of murine immune response genes has been thoroughly reviewed many times throughout the years (McDevitt and Benacerraf, 1969; Benacerraf and McDevitt, 1972; Benacerraf and Katz, 1975; Benacerraf, 1975; Benacerraf and Germain, 1978; Berzofsky, 1980a; Dorf, 1981; R. Schwartz, 1982; Klein and Nagy, 1982). However, the recent developments in cellular and molecular cloning have moved the problem of *Ir* gene function to the biochemical level. Thus, it seemed appropriate at this time to attempt to summarize this important area of cellular immunology so that structurally oriented biologists entering the field would have one place in which they could find a complete overview of the historical development and intellectual thought underlying the cell biology. As a consequence, this review is quite lengthy, yet at the same time does not consist of a complete compendium of all the published literature. Where possible, I have tried to cite the earlier reviews so that those readers wishing more complete bibliographies can find them. I hope my research colleagues will forgive me for any major omissions I might have made in this necessarily biased look back at the field of *Ir* genes.

II. Early Observations

A. A Single Genetic Locus Controls the Immune Response

The first laboratory to rigorously exploit the use of synthetic polypeptides to study the genetic control of the immune response in outbred and inbred animals was that of B. Benacerraf (see McDevitt and Benacerraf, 1969, for a review). The polymers studied were hapten conjugates of poly-L-lysine (PLL), such as dintrophenylated poly-L-lysine (DNP-PLL) and random copolymers of glutamic acid and lysine [poly$(Glu^{60},Lys^{40})_n$, abbreviated as GL]. When random bred Hartley strain guinea pigs were immunized with DNP-PLL or GL, anywhere from 10 to 40% of the animals tested responded

to the antigen (Kantor *et al.*, 1963). Responsiveness was measured both in terms of antibody formation, detected as precipitation or passive cutaneous anaphylaxis, and cell-mediated immunity, detected by delayed-type hypersensitivity (DTH) as assessed by skin testing. All responders produced both DTH and antibody titers to DNP-PLL and GL. All nonresponders produced neither immunological response to either antigen.

Such a clear distinction between responders and nonresponders allowed genetic studies to be cleanly executed (Levine *et al.*, 1963a,b; Levine and Benacerraf, 1965). When nonresponder Hartley strain guinea pigs were bred to each other, 100% of the offspring were nonresponders to DNP-PLL. When responder guinea pigs were bred, 82% were responders and 18% were nonresponders. Furthermore, two inbred strains were examined and found to differ absolutely in their responsiveness to DNP-PLL. All strain 2 guinea pigs responded to DNP-PLL while all strain 13 guinea pigs gave no response. Finally, a selective mating scheme, in which the homozygous nonresponder Hartley strain guinea pigs were mated to heterozygous responder guinea pigs (heterozygotes were animals that had produced nonresponder offspring), yielded 45% of the offspring that could respond to DNP-PLL and 55% that could not. This distribution was not significantly different by a Chi square test from the 50:50 distribution expected for the case of a single Mendelian dominant trait, and Levine and Benacerraf (1965) concluded that this was the case. However, the data are also consistent with the existence of two genetic loci, in which responsiveness is imparted when both loci possess high responder alleles. The expected value in this case is 42% responders if the three types of heterozygotes are present in equal frequency in the population. Nonetheless, the important concept that emerged from these studies was that the genetic control of the immune response was mediated by only a few genes.

More extensive genetic studies were carried out in the mouse by McDevitt and Sela (1965), using the synthetic copolymer, poly(Tyr,Glu)-poly (DL-Ala)--poly(Lys), abbreviated (T,G)-A--L. This molecule, which was synthesized by Sela *et al.* (1962), is a branched chain copolymer in which a poly-L-lysine backbone is extended from its ϵ amino side chain groups with a mixture of D- and L-alanine. This molecule (A--L) is then reacted with a mixture of the *N*-carboxyanhydrides of glutamic acid and tyrosine, which results in the addition of the two amino acids to the free α amino groups of the alanine side chains. Each step of the synthesis involves a chemical polymerization event that is random. Thus, every molecule is slightly different from every other one with regard to both composition and chain length. As a consequence, one can only specify the average values for molecular weight and molar composition. Most preparations have an average molecular weight of around 200,000 and a molar ratio for the amino acids of 2 Tyr:2–4 Glu:15–

20 DL-Ala:1 Lys, with an average poly-L-lysine backbone length of 100 lysines. However, it is important to keep in mind that each preparation is unique.

Mice were immunized with 10 μg of (T,G)-A--L in complete Freund's adjuvant in the hind foot pads and boosted 5 weeks later with an identical amount in saline. Ten days after the second injection, C57BL mice produced large amounts of antibodies that were detected in the serum with an antigen-binding assay (Farr assay) involving ^{125}I-labeled (T,G)-A--L and precipitation of the immune complexes with a rabbit anti-mouse immunoglobulin. In contrast, CBA mice immunized in exactly the same way produced 10-fold lower amounts of antibody. The (CBA×C57)F_1 was an intermediate responder to (T,G)-A--L, although the distribution of individual F_1 responses partially overlapped the distribution for the high responder C57 strain. A backcross of the F_1 mice to the low responder CBA parent produced offspring whose responses to (T,G)-A--L fell in the range encompassed by either the CBA or the F_1. A backcross of the F_1 mice to the high responder C57BL parent produced offspring whose range of responses to (T,G)-A--L encompassed either the C57BL or the F_1. These results were most compatible with the interpretation that a single major genetic factor controlled the antibody response to (T,G)-A--L and that responsiveness in the F_1 was partially dominant over nonresponsiveness. However, because of the quantitative nature of the data, a clear assignment of each animal to one of the three groups was not always possible. In addition, further analysis revealed occasional backcross mice with unexpectedly high or low titers. Thus, the possibility that one or more minor modifying genetic factors were also at work could not be excluded.

One important variable described by McDevitt and Sela (1965, 1967) for demonstrating immune response differences between strains was the dose of antigen used for immunization. If the C57 and CBA mouse strains were immunized with 1 μg of (T,G)-A--L, both strains were low responders. Only when the dose of antigen was raised to 10 μg per mouse did the 10-fold difference between the two strains emerge. Immunizing with 100 μg per mouse still revealed an easily detectable strain difference; however, the magnitude of the difference was not as great. Thus, it was clear from these studies that responsiveness was a relative trait that depended heavily on the dose of the immunologic challenge.

The most convincing evidence that only a single genetic locus regulated the immune response emerged from further studies in the guinea pig by Bluestein *et al.* (1971a,b) using the synthetic polypeptides, poly(Glu^{50},Tyr^{50})$_n$ (GT), and poly(Glu^{60},Ala^{40})$_n$ (GA). Inbred strain 13 guinea pigs immunized with 500 μg of GT in complete Freund's adjuvant all responded to the antigen, as measured by delayed-type hypersensitivity skin testing or anti-

body formation. The average percentage of antigen bound by the sera of all strain 13 animals tested was 42.7. In contrast, all inbred strain 2 guinea pigs when immunized in the same way did not respond at all to the antigen when challenged by skin testing and yielded sera whose average percentage of antigen bound (4.6 ± 1.2) was not significantly different from that of unimmunized controls. Thus, the strain 13 was designated a responder to GT while the strain 2 was designated a nonresponder.

All $(13 \times 2)F_1$ guinea pigs tested (six) responded to GT on skin testing after immunization, and the percentage of antigen bound (19.8×1.5) was intermediate between the responder and nonresponder levels. Backcross of the F_1 to the nonresponder strain 2 parent produced 17 offspring, 9 of which responded to GT on skin testing (53%) and 8 of which did not (47%). The 8 nonresponders had a mean percentage of antigen bound of 3.5, equivalent to that of the nonresponder strain 2 animals. The 9 responder animals had a mean percentage of antigen bound of 32.9, intermediate between the levels observed for the strain 13 responders and the $(13 \times 2)F_1$ hybrids. The authors concluded from these results that the GT-specific *Ir* gene was a single dominant autosomal locus.

The experiments were unambiguous in support of a single genetic locus. Responders and nonresponders could be cleanly distinguished and the expected 50:50 ratio in the backcross was obtained. However, the question of dominance was not clearly established, in particular because of the quantitative data on antibody formation. Codominant expression would predict a mean percentage of antigen bound of 23.6. The F_1 result of 19.8 supports the idea of codominance, while the responder population from the $F_1 \times$ strain 2 backcross, which should also be $(13 \times 2)F_1$ heterozygotes, suggested a partially dominant situation. We now know the correct interpretation is codominant expression. However, we will save until later a discussion of the factors that lead to the results of partial dominance seen by both Benacerraf and McDevitt when we review the phenomena of gene complementation and unique F_1 Ia molecule-specific T cell clones (Section V,B).

B. Antigen Specificity of the Genetic Regulation

The first scientists to discover that *Ir* genes regulated the antibody response in an antigen-specific manner were McDevitt and Sela (1965, 1967). In working with the branched chain synthetic polypeptides, they noted that if histidine were substituted for tyrosine, to produce (H,G)-A--L instead of (T,G)-A--L, the genetic control completely reversed. The CBA mouse was shown to be a responder to (H,G)-A--L, while the C57BL mouse was shown to be a nonresponder. In subsequent experiments, when a phenylalanine was substituted for a tyrosine, (P,G)-A--L, it was found that both strains responded equally well, while if the aromatic amino acid was deleted, leav-

ing only glutamic acid residues attached to the DL-Ala side chains, G-A--L, then neither strain responded. Thus, manipulating a single amino acid of the antigen, even in as subtle a way as changing only a hydroxyl group (Tyr versus Phe), could influence the immune response.

In the guinea pig similar results were obtained with the linear synthetic polypeptides, GA and GT(Bluestein *et al.*, 1971a,b). Strain 2 was a responder when glutamic acid was polymerized with alanine (GA), while strain 13 was a nonresponder. In contrast, when glutamic acid was polymerized with tyrosine (GT), the genetic control reversed, i.e., strain 13 was now the responder while strain 2 was the nonresponder.

In general, the antigen specificity of *Ir* gene control was so exquisite that no common structural properties were discernible in the antigens that might explain the chemical basis of the immunologic regulation. However, there was one striking and important exception to this observation and that was the poly-L-lysine system in guinea pigs. Levine *et al.* (1963a) discovered quite early that outbred guinea pigs that did not respond to DNP-PLL would also not respond to a variety of other haptens conjugated to PLL. These included the haptens benzylpenicilloyl (BPO), *p*-toluene sufonyl (Tosyl), and dimethylaminonaphthalene sulfonyl (DMANS). Similarly, outbred guinea pigs that did respond to DNP-PLL were capable of responding to all the hapten-PLL conjugates. The interesting observation was that the responder animals could discriminate among three of the four antigens. For example, responders immunized with $Tosyl_{25}$-PLL gave a positive delayed hypersensitivity reaction when skin tested with $Tosyl_{25}$-PLL but were negative when skin tested with BPO_{21}-PLL or DNP_{20}-PLL. Similarly, responders immunized with BPO_{21}-PLL gave a positive skin test only with BPO_{21}-PLL. These results demonstrated that at one level the immune system could recognize each of the hapten conjugates as a unique entity (DTH response), but that at another level, the step at which *Ir* genes exert their influence on immunogenicity, these conjugates were all seen as one family. Since the poly-L-lysine backbone appeared to be the structural element shared by these conjugates, recognition of PLL was postulated to be the critical step in *Ir* gene control.

C. Early Models of *Ir* Gene Function

1. Models

In order to explain the antigen-specific nature of immune response regulation by a single genetic locus, three different hypotheses were put forth by the early investigators. The first, which was suggested by Levine and Benacerraf (1964), postulated that *Ir* gene products were involved in antigen metabolism prior to recognition by lymphocytes. It was known from the

work of a number of investigators that D amino acid copolymers were poorly immunogenic compared to their equivalent L amino acid copolymers in both mice and guinea pigs, and that this correlated with the ability of the polymers to be degraded by cellular proteases (Sela, 1966). Thus, it was suggested that the difference between responders and nonresponders might be the presence in the responders of a single essential metabolic enzyme. This molecule would be antigen specific in its recognition; for example, it would recognize the poly-L-lysine backbone and either cleave it to a more immunogenic form or bind it to another cellular molecule (e.g., an RNA) to create a processed form of the antigen that was capable of inducing an immune response (Gottlieb and Schwartz, 1972). Such concepts became known as antigen-processing models. To test this idea, Levine and Benacerraf (1964) compared responder and nonresponder guinea pigs for their ability to take up and degrade, both *in vivo* and *in vitro*, radioactively labeled poly-L-lysine, poly-D-lysine, and their succinylated and haptenated derivatives. No differences were found, suggesting that metabolic breakdown alone could not be the critical difference between responder and nonresponder animals.

The second model, championed predominantly by Cinader (1963), postulated that nonresponsiveness stemmed from tolerance induction to self macromolecules. The major concept was that each individual possessed a unique set of macromolecules because of the genetic polymorphisms that exist within the species. If one form of a pleiomorphic determinant on a macromolecule happened to structurally mimic a determinant on a foreign antigen, then tolerance to the self determinant would preclude that individual from responding to the foreign determinant (nonresponder). In contrast, another individual of the same species, who possessed a different allelic form of the self determinant, would be able to respond to the foreign determinant (responder). Thus, in this model the *Ir* gene would encode a particular self macromolecule. The major argument against this model was that responsiveness was dominant or codominant in the F_1. A tolerance model (in this form) would predict that nonresponsiveness would be dominant, because the F_1 should inherit and express the self macromolecule coded for by the nonresponder parental genome, thus leading to deletion of the response against the foreign determinant.

The third model, espoused mainly by McDevitt (1968), postulated that *Ir* genes regulated the structure of the antibody molecules elicited by the antigen. This was suggested largely because of the exquisite antigen specificity of the *Ir* gene control and because antibodies were the only molecules of the immune system known at the time to be capable of performing such fine discriminations. However, a number of experiments carried out by both Benacerraf's group and McDevitt and Sela argued strongly that this model was not correct (see McDevitt and Benacerraf, 1969, for a review). First,

early genetic linkage studies on (CBA×C57)F_1 × CBA backcross mice, which examined the segregation of immune responsiveness with the *Igh-1*^{b}-encoded allotypic determinants on C57BL IgG_{2a} anti-(T,G)-A--L antibodies, demonstrated that the *Ir* genes were not linked to the immunoglobulin heavy chain gene complex. Second, antibodies raised against (T,G)-A--L in C57 mice were found to crossreact with (H,G)-A--L, even though C57 mice could not respond when immunized with (H,G)-A--L. Similarly, anti-(H,G)-A--L antibodies cross-reacted with (T,G)-A--L. Thus, the antibodies appeared to be less specific in their binding than the *Ir* gene control.

2. *The "Schlepper" Experiment*

Probably the most influential experiment arguing against the participation of *Ir* genes in antibody specificity was the so-called "schlepper" (German for carrier) experiment. This was first performed with DNP-PLL in guinea pigs by Green *et al.* (1966). If the positively charged DNP-PLL molecules were electrostatically coupled to negatively charged human serum albumin (HSA), ovalbumin (OVA), bovine serum albumin (BSA), or acetylated BSA, and the resulting complex used to immunize nonresponder guinea pigs, then the animals made antibodies to DNP-PLL. The amount of antibody produced, as measured by equilibrium dialysis, was equivalent to that produced in responder guinea pigs. Furthermore, the specificity of the antibody produced, as measured by a greater fluorescence quenching with DNP-PLL than with ϵ-DNP-L-lysine, was the same for both responders and nonresponders. In order to elicit anti-DNP-PLL antibodies in the nonresponder animals, the carrier molecule itself had to be immunogenic in these animals. Thus, the complex of DNP-PLL with the self macromolecule, guinea pig albumin (DNP-PLL:GPA), elicited little (4 animals) or no (8 animals) antibodies. Similarly, nonimmunogenic, negatively charged, nonprotein polymers, such as dextran sulfate, polystyrene sulfonate, and carboxymethylcellulose, when complexed to DNP-PLL, elicited no antibodies. All of these molecules elicited normal levels of anti-DNP antibodies in responder guinea pigs, showing that the nonimmunogenic carriers were not nonspecifically suppressive for antibody responses. Finally, induction of tolerance to BSA in nonresponder guinea pigs by administering high doses of the molecule prevented the animals from making anti-DNP-PLL antibodies when challenged with DNP-PLL:BSA.

In contrast to their ability to make anti-DNP-PLL antibodies, nonresponder guinea pigs immunized with DNP-PLL:foreign albumin complexes were still not able to give a DTH reaction when skin tested with DNP-PLL. They did give a positive DTH reaction when challenged with the foreign albumin. Thus, DNP-PLL appeared to behave like a hapten in the nonres-

ponder animals. It was not immunogenic by itself, but if coupled to a carrier molecule, it could elicit antibodies specific for itself. This, in turn, suggested that responder guinea pigs must be capable of recognizing part of the PLL molecule as a carrier determinant, whereas the nonresponder guinea pigs behaved as if they had a specificity defect, i.e., they were unable to recognize the PLL carrier determinant. This specificity defect did not appear to be in the B cell repertoire because it could be by-passed at the antibody level by complexing the antigen to an immunogenic carrier. This observation, plus the failure to overcome the defect in DTH, suggested that the problem was located at an earlier step in the immune response, one that was essential for both DTH and antibody formation.

Since 1966 this seminal observation has been confirmed repeatedly by investigators using many different antigens (McDevitt, 1968; Gershon *et al.*, 1973; Kapp *et al.*, 1974a; and Debre' *et al.*, 1975a). However, it should be noted that a few exceptions have been found. Occasionally an antigen would elicit a DTH response even though it could not sensitize the animal. For example, Benacerraf *et al.* (1963) showed that azobenzene arsonate (ABA) coupled to poly(D-Glu^{60},D-Ala^{30},D-Tyr^{10})$_n$, a GAT copolymer composed of D amino acids, would not prime guinea pigs to give a DTH response to ABA-L-GAT or ABA-D-GAT. However, if the guinea pigs were primed with ABA-L-GAT, then the ABA-D-GAT could elicit a DTH response. Similar observations were made in the mouse for the nitrophenyl acetyl (NP) hapten coupled to poly(Glu^{56},Lys^{35},Phe^{9})$_n$ (GLPhe) (Weinberger *et al.*, 1979). The significance of these few exceptions still is not clear. Possibly they represent the presence of hapten-specific T cells (see Section VI,B,2,c).

D. *Ir* Gene Expression Requires Immune Responses Involving T Lymphocytes

The realization that the early step of *Ir* gene control might be at the level of T lymphocyte antigen recognition only came several years after the experiments described above, when thymus-derived (T) lymphocytes were, in fact, first discovered. The bone marrow and thymus reconstitution experiments of Claman *et al.* (1966) and Mitchison's classical hapten-carrier experiments (Mitchison, 1971a,b) paved the way for a two lymphocyte model, each with specificity for different parts of the antigen. In the context of this model, the DNP-PLL experiments were subsequently interpreted in terms of a requirement for antigen bridging between a B cell specific for ϵ-DNP-Lys-(Lys)$_x$ and a T cell specific for a carrier determinant [e.g. (Lys)$_7$] present on the PLL backbone (Benacerraf *et al.*, 1967). Because nonresponder guinea pigs appeared to lack the ability to recognize the PLL carrier determinant, the *Ir* gene defect was thought to lie in the T cell repertoire, where clones with this specificity were assumed to be missing. The mechanism by

which this occurred was postulated to be through the encoding of T cell antigen-specific receptors by the *Ir* genes (Benacerraf and McDevitt, 1972). Thus, the responder strain 2 guinea pig would possess a series of *Ir* loci, one of which encoded a receptor specific for PLL, while the nonresponder strain 13 guinea pig would lack such a gene, although it would possess another gene that encoded a receptor specific for GT. The "schlepper" experiment would work by providing the nonresponder animal with a carrier determinant that its T cell repertoire could recognize. This would allow T cell help to take place and stimulate B cells specific for DNP-Lys-$(Lys)_x$ or presumably any other hapten on PLL, since the B cell repertoire was postulated to be totally intact.

Evidence to support the idea that *Ir* genes influenced mainly T cell responses came from several sources (reviewed in Benacerraf and Katz, 1975). First, immunological assays involving only T lymphocytes (and not B lymphocytes) were shown to be affected by *Ir* genes. In addition to the DTH assay described above, which has been shown to be T cell-mediated, *in vitro* assays involving T cell proliferation were shown to be strongly influenced by *Ir* gene effects. For example, Schwartz and Paul (1976) developed a murine T cell proliferation assay using nylon wool column-purified T lymphocytes from the peritoneal cavity. When C57BL/10 mice were immunized to (T,G)-A--L, their T cells proliferated in vitro if stimulated with (T,G)-A--L but not with (H,G)-A--L. B10.BR mice (identical *Ir* genes to the CBA mouse) when immunized with (T,G)-A--L were nonresponders, i.e., they did not proliferate at all to (T,G)-A--L. In contrast, when B10.BR mice were immunized with (H,G)-A--L, their T cells proliferated *in vitro* if stimulated with (H,G)-A--L, but not with (T,G)-A--L. Finally, when C57BL/10 mice were immunized with (H,G)-A--L, they did not respond. These *in vitro* results were identical to the *in vivo* observations of McDevitt and Sela (1965, 1967), on the immunogenicity of these two antigens in C57 and CBA mice, and demonstrated that *Ir* gene control of T cell proliferation was the same as that for antibody formation.

One apparent difference in the antibody and T cell proliferation results was the difference in cross-reactivity of the antigens in the two assay systems. At the T cell level no cross-stimulation was observed *in vitro*. In contrast, at the antibody level, anti-(T,G)-A--L antibodies would react with (H,G)-A--L and vice versa. This difference, however, merely serves to emphasize the site of *Ir* gene expression. If instead of examining antibodies, the products of stimulated B cells, helper T cells were assayed, then analogous results to the proliferation assay were observed. Thus, when McDevitt and Sela (1967) primed (CBA × C57BL)F_1 mice with (T,G)-A--L and then tried to boost the animals with (H,G)-A--L, no secondary antibody response was detected. The failure of this cross-priming experiment demonstrated that

the exquisite antigenic fine specificity (recognition of tyrosine vs histidine) was at the level of the helper T cell not the B cell.

McDevitt (1968) also carried out a "schlepper" experiment that demonstrated the same point. CBA mice were immunized with (T,G)-A--L (negatively charged) electrostatically coupled to methylated bovine serum albumin (MBSA) (positively charged). A primary antibody response was detected. At 5 weeks the mice were boosted with either (T,G)-A--L alone or (T,G)-A--L complexed to MBSA. The complex elicited a secondary response whereas the (T,G)-A--L alone did not. Thus, similar to the elicitation of DTH in guinea pigs, restimulation of helper T cells required the presence of carrier determinants (MBSA) that the CBA mice could recognize. (T,G)-A--L alone did not contain these determinants, although it did contain determinants that CBA B cells could recognize.

Another piece of evidence supporting the critical role of T cell responses in distinguishing responder and nonresponder mice came from the studies of Grumet (1972) on the antibody responses to (T,G)-A--L administered only in saline, i.e., in the absence of Freund's complete adjuvant. Under these conditions it could be shown that both high and low responders made comparable primary antibody responses to (T,G)-A--L and that all of the antibody was IgM (2-mercaptoethanol sensitive). Only on boosting did the difference between high and low responders emerge. The low responders continued to produce low levels of IgM antibodies, whereas the high responders converted to making IgG antibody (2-mercaptoethanol resistant) in large amounts. Thus, the principal difference between the two strains was the IgG memory response.

That this memory was dependent on an intact T cell immune response was shown by thymectomy experiments. Mitchell *et al.* (1972) either thymectomized neonatal responder mice or thymectomized adult mice of this type, followed by 750 rads of X-irradiation and reconstitution with syngeneic bone marrow (so called ATXBM mice). Both procedures severely depleted the animals of T lymphocytes. When such animals were immunized with (T,G)-A--L in saline, they produced the IgM primary response normally seen in both high and low responder mice. However, when boosted with (T,G)-A--L in saline (or adjuvant), the T cell-depleted high responder mice did not respond with an IgG secondary response, whereas sham thymectomized mice did produce an IgG response. Similar results were obtained when ATXBM low responder CBA mice were immunized with (T,G)-A--L complexed to MBSA. Thus, elimination of T cells converted the high responders to a low responder phenotype and prevented MBSA from circumventing the *Ir* gene regulated defect. It was concluded from these studies that the primary antibody response to (T,G)-A--L in saline was a T cell-independent response and not subject to *Ir* gene control, whereas the secondary IgG mem-

ory was a T cell-dependent immune response that was subject to *Ir* gene control. The effect of the *Ir* gene in nonresponder mice was considered to be a functional or cognitive lesion in the T cell population with respect to the ability of these cells to recognize a carrier determinant on (T,G)-A--L, i.e., they appeared to be functionally "thymectomized" to this antigen.

Another experiment that supported this point of view was the ability of an ongoing graft-versus-host reaction to bypass *Ir* gene control in nonresponders. Ordal and Grumet (1972) immunized (C3H × C3H.Q)F_1 nonresponders with (T,G)-A--L at the same time that they administered nonresponder parental C3H spleen and lymph node cells. The latter cells recognized as foreign the $H\text{-}2^q$-encoded molecules on the F_1 hosts' tissues and mounted an immunological reaction against them. This graft-versus-host reaction produced an allogeneic effect, which was shown by Hamoaka *et al.* (1973) to provide antigen-nonspecific stimuli that could substitute for antigen-specific T cell help. In this case, it allowed the low responder B cells to produce high levels of anti-(T,G)-A--L antibody and to switch from IgM to IgG, both characteristics of high responder mice. Thus, the low responders appeared to have normal B cells capable of making anti-(T,G)-A--L antibodies, but to lack helper T cells specific for (T,G)-A--L. The exact mechanism by which the allogeneic effect can substitute for antigen-specific T cell help is still unknown, but presumably involves lymphokines secreted by the responding alloreactive T cells.

Finally, as the genetic control of the immune response to more and more antigens was explored, an important pattern emerged which also supported the notion that T cell responses were essential for the manifestation of *Ir* gene regulation. All antibody responses that were found to be under *Ir* gene control were also found to be to thymic-dependent antigens, i.e., to require helper T lymphocytes for a full response, similar to the results with (T,G)-A--L described above (for a review see Benacerraf, 1975). In contrast, antibodies to polysaccharide (Blomberg *et al.*, 1972; Amsbaugh *et al.*, 1972) and polynucleotide (Scher *et al.*, 1973) antigens, which could be elicited in the absence of a thymus, did not appear to be influenced by *Ir* genes. These latter antigens were genetically regulated by genes linked to the immunoglobulin heavy chain allotype locus or by genes found on the X chromosome (for a review see Berzofsky, 1980a). Thus, it was concluded that T cells play an obligate role in *Ir* gene-controlled responses.

E. *Ir* Genes Are Genetically Linked to the Major Histcompatibility Complex

One of the most important advances in our understanding of *Ir* genes occurred when McDevitt and Tyan (1968) mapped these genes to the major histocompatibility complex (MHC). In the process of doing adoptive transfer

experiments to localize the cellular sites of expression of *Ir* genes (see below), they discovered that when strains were matched at the MHC, which was done in order to minimize graft-vs-host reactions after cell transfer, the animals displayed the same immune response phenotype. In particular, a careful examination of the immune responses of *H-2* (murine MHC) congenic mice, developed by Snell and Stimpfling, demonstrated that only differences in *H-2* genes were required to see differences in immune response phenotypes (McDevitt and Chinitz, 1969). For example, C3H.SW mice are C3H mice bearing genes of the $H\text{-}2^b$ haplotype instead of their normal $H\text{-}2^k$ haplotype genes. When C3H.SW mice were immunized with (T,G)-A--L they gave a substantial antibody response, more akin to the response of C57 mice, which also bear the $H\text{-}2^b$ haplotype, than to C3H mice, which share all genes other than those in *H-2*. Similarly, immunization of C3H.SW mice with (H,G)-A--L produced no detectable antibodies, i.e., they were nonresponders like C57 mice. In contrast, C3H mice were good responders to (H,G)-A--L. Identical results were obtained when the congenic pairs B10.BR-C57BL/10 and A/J-A.BY were compared. Overall, these results suggested that the ability to respond to these two antigens could be bred into or out of a particular strain by selecting only for the $H\text{-}2^b$ or $H\text{-}2^a$ or $H\text{-}2^k$ haplotypes.

A formal proof of the genetic linkage of *Ir* genes to the MHC was performed using the segregating population in a backcross to the low responder parent. $(CBA \times C57)F_1 \times CBA$ backcross mice were examined both for their immune response to (T,G)-A--L and their possession of the $H\text{-}2^b$ haplotype, the latter being tested by typing peripheral blood cells from the mice with anti-$H\text{-}2^b$ antisera. Of the back cross mice 16/38 (42%) were responders to (T,G)-A--L. Of these, 15/16 (94%) typed positive for expression of $H\text{-}2^b$ gene products. Conversely, of the 22 nonresponder mice, none of them typed positive for $H\text{-}2^b$ gene products. Thus, the inheritance of the $H\text{-}2^b$ genes was tightly linked (1 potential recombinant out of 38 mice) to the *Ir* gene(s) that controlled the ability to respond to (T,G)-A--L. Similar results were obtained by McDevitt and Tyan (1968), linking the *Ir* gene for (H,G)-A--L to the $H\text{-}2^k$ haplotype in the mouse and by Ellman *et al.* (1970), linking the *Ir* gene for DNP-PLL to the strain 2 histocompatibility genes in the guinea pig.

Fine structure genetic mapping of the *Ir-1* gene controlling the antibody response to the branched chain synthetic polypeptides was carried out by McDevitt *et al.* (1972) in collaboration with a number of the well known mouse geneticists (Snell, Stimpfling, Shreffler, and Klein). These experiments utilized the *H-2* recombinant strains that had already been developed by the geneticists. The immune responses to (T,G)-A--L, and (Phe,G)-A--L were determined in 11 different strains bearing *H-2* recombinant chromosomes derived from cross-overs between the *H-2K* and *H-2D* loci of estab-

lished mouse strains, which themselves bore known *H-2* alleles and known *Ir-1* phenotypes. In all cases the *Ir* genes mapped between the *H-2K* and *S* loci, defining a new genetic region. For example, mice bearing the $H\text{-}2^a$ haplotype, such as the A strain, were high responders to (H,G)-A--L, whereas mice bearing the $H\text{-}2^b$ haplotype, such as C3H.SW and C57BL/10, were nonresponders. The recombinant mouse strain B10.A(4R) possesses the *a* allele at the *H-2K* locus but *b* alleles at the *S* and *H-2D* loci, i.e., the genetic recombination occurred between *H-2K* and *S*. This strain responded to (H,G)-A--L in a manner similar to the high responder A strain suggesting that the *Ir* gene for the (H,G)-A--L response mapped to the left of (centromeric to) the S locus. The two strains which established the centromeric border were the DBA/1 and the AQR. DBA/1, bearing the $H\text{-}2^q$ haplotype, was a nonresponder to (H,G)-A--L. In contrast, the AQR recombinant, which possesses the *q* allele at the *H-2K* locus, but *a* alleles in the *S* and *D* regions of *H-2*, was a responder to (H,G)-A--L. This result demonstrated that the genetic cross-over event took place between the *H-2K* locus and the *Ir* locus, thus mapping the latter to the right of (telomeric to) *H-2K*.

As additional antigens were studied, it soon became clear that more than one *Ir* gene could be genetically distinguished between *H-2K* and *S*. The first antigen for which this was accomplished was the BALB/c ($Igh\text{-}1^a$) IgG_{2a} myeloma protein, MOPC 173. Lieberman and Humphrey (1971, 1972) demonstrated that the antibody response to the IgG_{2a} myeloma protein was controlled by genes linked to the *H-2* complex. Mice bearing the $H\text{-}2^b$ haplotype were responder strains and mice bearing the $H\text{-}2^a$ haplotype were nonresponder strains. However, in using the recombinant strains to map the genes within the *H-2* complex, Lieberman *et al.* (1972) found that the B10.A(4R) ($K^aIr\text{-}1A^aS^bD^b$) and B10.A(5R) ($K^bIr\text{-}1A^bS^aD^a$) strains were both responders to the IgG_{2a} myeloma protein. This result suggested that there was a second genetic locus to the right of (telomeric to) the *Ir-1A* gene regulating the (H,G)-A--L response. This new gene was designated *Ir-1B*.

Overall, these mapping experiments demonstrated that several *Ir* genes were located between the two known histocompatibility loci, in particular in the region between *H-2K* and *S*. Because these *Ir* genes were the first to be mapped to this area of the *H-2* complex, the region was designated the *I* region. Genes mapping in the same location as *Ir-1A* were grouped together as the *I-A* subregion and genes mapping in the same location as *Ir-1B* were grouped together as the *I-B* subregion. Subsequent studies mapped *Ir* genes to two more subregions, designated *I-E* and *I-C*. The molecular reality of this phenotypic mapping will be discussed later (see Section VI,C,4,d).

The major impact of the mapping of *Ir* genes to the MHC was to focus attention on the nature of the *Ir* gene product. Niels Jerne, in a radical hypothesis published in 1971, suggested that the *Ir* gene products were the

histocompatibility molecules involved in graft rejection, but that their primary function was to serve as a driving force for diversification of the lymphocyte repertoire during development. In this model, *Ir* gene control was a secondary consequence of the selection and somatic mutational events that occurred in the thymus, i.e., the repertoire that developed in any given animal contained gaps or holes that would not allow it to respond to all antigens. In contrast, Benacerraf and McDevitt in 1972 postulated that the *Ir* genes encoded the antigen-specific T cell receptor. They focused mainly on the fact that the known histocompatibility molecules were cell surface proteins and that the *Ir* genes could be separated from them by recombination, i.e., no *Ir* genes mapped to *H-2K* or *D*. In addition, the existence of several separable genes within the *I* region controlling the response to different antigens or determinants on the same antigen suggested a unique family of genes with similar function. Finally, the exquisite fine specificity of the regulation of the immune response pushed them in the direction of a receptor molecule on the lymphocyte surface.

The essential difference between these two models is that Benacerraf and McDevitt postulated that *Ir* genes influence the specificity of the response by encoding receptor molecules, whereas Jerne postulated they influence the immune response by encoding molecules that are recognized by receptor molecules (the latter being encoded elsewhere). Although it is fair to say that neither model anticipated the phenomenon of MHC restriction, which is the underlying link between the T cell receptor and the MHC-encoded molecules, the Jerne version appears today to have been far closer to the truth. However, it took a number of years before this was recognized.

F. Generality of *Ir* Gene Control of Responses to Polypeptides

During the ensuing years numerous *Ir* genes were described that controlled the immune response to a variety of different antigens. In most cases where mapping was done, the genes were located in the major histocompatibility complex of the species, and for the mouse in the *I* region of the MHC. In each case, the antigen appeared to have a unique pattern of responsiveness and nonresponsiveness among the inbred strains of mice bearing independent *H-2* haplotypes. In other words, each antigen had its own signature, presumably reflecting in part the biochemical composition of the molecule and in part the discriminating power of the immune system. A summary of such strain distributions of responsiveness for a selected sample of antigens is shown in Table I.

Some antigens were only immunogenic in one or a few strains. For example, the synthetic polypeptide, poly(Glu^{57},Lys^{38},Pro^{5})$_n$ (GLPro) was immunogenic only in mice possessing the $H\text{-}2^s$ haplotype, while the sequential

TABLE I

THE RESPONSIVENESS OF MICE POSSESSING DIFFERENT MHC HAPLOTYPES TO A VARIETY OF SYNTHETIC AND NATURAL POLYPEPTIDE ANTIGENS[a]

Antigen	a	h4	b	i5	d	g2	f	j	k	p	q	r	s	t4	u	v	Assay
Poly(Glu^{60},Ala^{40})$_n$	H	—	H	—	H	—	H	N	H	N	N	H	H	—	H	H	A
Poly(Glu^{40},Ala^{60})$_n$	—	—	H	—	H	—	H	—	H	—	N	—	H	—	—	—	A
Poly(Glu^{40},Ala^{60})$_n$	—	—	H	H	H	—	H	N	H	N	N	N	N	—	N	N	P
Poly(Glu^{50},Tyr^{50})$_n$	N	—	N	—	N	—	N	N	N	N	N	N	N	—	N	N	A
Poly(Glu^{60},Lys^{40})$_n$	N	—	N	—	N	—	N	N	N	N	N	N	N	—	N	N	A
Poly(Glu^{60},Ala^{30},Tyr^{10})$_n$	H	H	H	H	H	—	H	N	H	N	N	H	L/N	—	H	H	A
Poly(Glu^{57},Lys^{38},Ala^{5})$_n$	I	I	L	L	H/I	—	H	H	H/I	I/L	H/I	I	H	—	—	—	A
Poly(Glu^{57},Lys^{38},Tyr^{5})$_n$	N	—	N	I	H	N	N	H	N	N	N	H	N	N	H	N	A
Poly(Glu^{51},Lys^{34},Tyr^{15})$_n$	N	—	N	I	H	—	N	H	N	N	I	H	N	N	H	N	A
Poly(Glu^{53},Lys^{36},Phe^{11})$_n$	N	—	N	H	H	N	L	H	N	H	H	H	N	H	H	N	A
Poly(Glu^{53},Lys^{36},Phe^{11})$_n$	N	N	N	H	H	—	L	H	N	—	H	H	N	H	H	N	P
Poly(Glu^{57},Lys^{38},Pro^{5})$_n$	N	—	N	—	N	—	N	N	N	N	N	N	H	—	N	N	A
Poly(Glu^{56},Lys^{37},Ser^{7})$_n$	H	—	N	—	N	—	H	N	H	N	N	N	N	—	N	N	A
Poly(Glu^{55},Lys^{35},Leu^{10})$_n$	L/N	—	N	I/L	I/L	N	N	H	L/N	N	N	H	N	H	I/L	H	A
(Tyr,Glu)-DL-Ala--Lys	L	L	H	H	I	—	L	L	L	L	L	L	L	—	—	—	A
(His,Glu)-DL-Ala--Lys	H	H	L	L	I	—	L	L	H	L	L	—	L	—	—	—	A
(His,Glu)-DL-Ala--Lys	H	H	N	N	N	—	—	—	H	—	—	—	N	—	—	—	P
(Phe,Glu)-DL-Ala--Lys	H	H	H	H	H	—	H	H	H	H	H	—	L	—	—	—	A
Poly-(Tyr-Ala-Glu-Gly)$_n$	N	N	H	H	N	N	H	—	N	N	N	H	N	—	—	—	A
Poly-(Tyr-Glu-Ala-Gly)$_n$	N	N	H	H	N	N	N	N	N	N	N	N	N	—	—	—	A
TNP-Poly-[Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$]$_n$	N	—	N	N	H	—	—	—	—	—	N	—	—	—	—	—	A
MOPC 467:BALB/c IgA	H	H	N	N	N	—	—	—	H	H	N	H	H	—	N	N	A
TEPC 15:BALB/c IgA	H	H	N	N	N	—	—	—	H	—	—	L	L/N	I/L	—	—	P
MOPC 173:BALB/c IgG_{2a}	N	H	H	H	N	—	—	—	N	H	N	H	H	—	—	H	A
H-2.2 alloantigen on D^b	N	—	N	H	H/N	—	—	—	H	—	—	—	—	—	—	—	A
Thy 1.1 alloantigen	L	L	L	L	L	L	L	—	H	L	L	—	L	—	—	—	A

(*continued*)

TABLE I (*Continued*)

Antigen	a	h4	b	i5	d	g2	f	j	k	p	q	r	s	t4	u	v	Assay
Sex limited protein(Slp)	T	H	N	N	T	—	H	T	H	T	H	—	T	—	T	N	A
Ea-2.1 erythrocyte antigen	—	—	N	—	—	—	—	—	—	—	—	H	—	—	—	—	A
Mouse thyroglobulin	H	H	L	L	I	I	L	—	H	—	H	H	H	H	—	—	A
Pigeon cytochrome *c*	H	N	N	N	N	—	L	N	H	N	N	L	L	H	N	H	P
Bovine insulin	N	N	H	H	H	—	—	—	N	—	N	N	N	—	N	H	A
Ovine insulin	H	L	L	L	—	—	—	—	—	—	—	—	—	—	—	—	P/A
Porcine insulin	—	—	N	—	H	—	—	—	N	—	N	N	—	—	—	—	A
Sperm whale myoglobin	I	L	L	I	H	H	—	—	L	—	L	—	H	H	—	—	A
Hen egg lysozyme	H	—	L	—	I	—	—	—	H/I	—	H	—	—	—	—	—	A
Hen egg lysozyme	H	H	N	N	N	—	H	L	H	H	L	N	N	N	—	—	P
Staphylococcal nuclease	H	L	L	L	H	—	H	H	H	—	H/L	H	H	—	—	—	P
Staphylococcal nuclease	H	L	L	L	H	—	—	—	H	—	L	—	H	—	—	—	A
Calf procollagen peptide	H	—	H	—	L	—	—	—	H	—	L	—	L	I/L	—	—	A
Collagen (calf type 1)	L	L	H	H	I/L	—	H	L	L	L	N	I	H	—	I/L	—	A
Lactate dehydrogenase A	H	—	L	—	H	—	H	H	H	H	H	H	H	—	H	H	A
Lactate dehydrogenase B	L	—	H	—	H	—	H	I	L	H	H	I	H	—	I	I	A
Lactate dehydrogenase B	N	H	H	H	H	H	H	N	L/N	H	H	H	H	H	H	H	P
Ragweed pollen extract	H	H	L	L	H	—	L	L	H	H	—	—	—	—	L	—	A
Low dose ovomucoid	L	—	H	—	I	—	H	H	L	H	H	H	I	—	—	—	A
Low dose ovalbumin	H	—	L	—	L	—	L	—	H	—	L	—	L	L	—	—	A
Torpedo acetylcholine receptor	—	N	H	H	L	L	I	H	I	L	H	H	I	—	—	—	P

[a] Symbols at the tops of the columns are the MHC haplotype designations. The h4 and g2 represent haplotypes from strains bearing recombinant chromosomes between $H\text{-}2^b$ and $H\text{-}2^a$ or $H\text{-}2^d$, respectively. The i5 haplotype is the reciprocal recombination of $H\text{-}2^b$ and $H\text{-}2^a$, and the t4 haplotype a recombination of $H\text{-}2^s$ and $H\text{-}2^a$. The letters in each column are abbreviations for responsiveness and stand for H, high responder; I, intermediate responder; L, low responder; N, nonresponder; T, tolerant to this self protein; —, not determined. Two letters in one box implies either different results from two laboratories or from two strains with the same MHC haplotype but different non-MHC backgrounds. The assays are grouped under two categories, T cell proliferation (P) and antibody responses (A). The latter could be any form of serum determination such as a Farr assay, complement fixation assay, hemagglutination assay, or passive cutaneous anaphalaxis, or alternatively a plaque-forming cell response. The data were obtained from the following reviews: Benacerraf and Katz (1975), Dorf (1978), Krco and David (1981), and Longo and Paul (1982). Primary sources were checked whenever there was a difference among the reviews.

copolymer poly(Tyr-Glu-Ala-Gly)$_n$ was only immunogenic in mice possessing the *H-2*^{b} haplotype. Other antigens were immunogenic in many strains. For example, only mice possessing the *H-2*^{j}, *H-2*^{p}, and *H-2*^{q} haplotypes failed to respond to poly(Glu^{60},Ala^{40})$_n$ while only mice of the *H-2*^{s} haplotype failed to respond to (Phe,G)-A--L. In general, there were three types of antigens whose responses were under detectable *Ir* gene control: (1) synthetic polymers composed of only a few amino acids, (2) allogeneic and xenogeneic proteins, which presumably differed from the animals own analogous proteins by a small number of amino acids, and (3) complex protein antigens such as ovomucoid and ovalbumin, which consist of multiple antigenic determinants. In the last group, immunization with low doses of antigen was often required to detect low responders, presumably because this created conditions where only the most immunogenic determinants were recognized.

In general, the more complex the antigen, the fewer the number of nonresponder strains, although striking exceptions such as poly(Glu^{60},Ala^{40})$_n$ should be noted. Thus, limited structural heterogeneity appeared to be a key factor in unmasking *Ir* gene control. This in turn suggested that the immune system, at some level, focuses on individual determinants on the antigen. Proof of this idea came from studies on protein antigens, which showed that distinct *Ir* genes control the responses to different sites on the same molecule. For example, in studying the immune response to sperm whale myoglobin, Berzofsky *et al.* (1979) discovered that two *H-2*-linked genes mapping in distinct subregions of the *I* region controlled both the antibody and T cell proliferative responses to this antigen. B10.D2 mice (*I-A*^{d}; *I-C*^{d}) were high responders, B10 mice (*I-A*^{b}; *I-C*^{b}) were low responders, and B10.A(5R) recombinant mice (*I-A*^{b}; *I-C*^{d}) were intermediate responders. In examining the nature of the intermediate response, Berzofsky dissected the antibody and T cell proliferative responses to sperm whale myoglobin by determining the binding of or proliferation to different cyanogen bromide-derived peptide fragments of myoglobin. Comparing the amino-terminal fragment, residues 1–55, with the carboxy-terminal fragment, residues 132–153, he found that B10.D2 mice responded to both fragments, B10 mice responded to neither fragment, and B10.A(5R) mice responded to fragment 1–55 but not fragment 132–153. Thus, the *d* allele of the *Ir* gene in *I-C*, either alone or in conjunction with the *b* allele of the *Ir* gene in *I-A*, controlled the recognition of a determinant on the 1–55 fragment, but had no effect on the recognition of a determinant on the 132–153 fragment. The response to the 132–153 part of the molecule was regulated only by *Ir* gene(s) in the *I-A* subregion.

These results demonstrated that different antigenic determinants on the same molecule can be controlled by different *Ir* genes. This suggests that the

failure to detect *Ir* gene control of complex antigens, such as the large protein antigens, is simply because the probability of recognizing a single determinant on the molecule approaches one when many different determinants exist. Thus, the thymic-dependent response to *all* polypeptide antigens is under *Ir* gene control. The masking of this control for the more antigenically complex molecules can be circumvented if one analyzes the response to individual determinants on the molecule.

III. Cellular Sites of Expression of *Ir* Genes

A. *Ir* Gene Influence on the Specificity of B Cell Responses

By 1972 it was clear that *Ir* genes expressed their functional effects in assays involving T lymphocytes, whether or not B lymphocytes were involved. However, whether the genes could also influence the specificity of B cell responses was not entirely clear. Evidence against *Ir* genes directly influencing B cell responses largely involved experiments in which the antigen-specific helper T cell defect was circumvented by allogeneic effects or "schlepper" molecules (see Section II,D). In these experiments, the B cells appeared to make a normal response, producing antibody of similar specificity to that produced by a responder B cell. In the most elaborate of these studies, M. Schwartz *et al.* (1978) raised site specific antiidiotypic antibodies against high responder (C3H.SW) anti-(T,G)-A- -L antibodies and showed that they reacted with a similar percentage (30%) of antibodies from low responder (C3H) mice immunized with (T,G)-A- -L coupled to methylated bovine serum albumin. Thus, B cells existed in the low responder with antigen receptors that were very similar or identical to the receptors on high responder B cells. The failure to stimulate these B cells was postulated to be the absence of a T cell receptor (*Ir* gene defect) specific for a carrier determinant on the molecule.

A logical extension of this hypothesis is that the presence of a carrier determinant on any molecule that is recognized by helper T cells should allow all B cell-specific (haptenic) determinants to be recognized, resulting in antibodies elicited against these determinants. However, several studies suggested that this was not always the case. In one early set of experiments, Bluestein *et al.* (1972) examined the fine specificity of antibodies against the synthetic copolymer, poly$(Glu^{60},Ala^{30},Tyr^{10})_n$ (GAT) in strain 2 and 13 guinea pigs by fractionating the antibodies into those that bound to poly$(Glu^{60},Ala^{40})_n$ (GA) and those that bound to poly$(Glu^{50},Tyr^{50})_n$ (GT). Strain 2 is a nonresponder to GT and a responder to GA, while strain 13 is the opposite, a responder to GT and a nonresponder to GA. When strain 2 was immunized with the GAT terpolymer, it produced antibodies that cross-

reacted with the GT copolymer alone. This result could be explained in terms of the "schlepper" concept in which T cells specific for GA determinants provided help for B cells specific for GT determinants. In contrast, when strain 13 was immunized with GAT, no antibodies were produced that cross-reacted with the GA copolymer. Strain 2, the high responder, did produce GA cross-reactive antibodies. Thus, in this case, the GT-specific helper determinants that the strain 13 could recognize did not allow effective presentation of GA determinants to the nonresponder B cells. This failure of the GT determinants in GAT to "schlepp" for GA determinants, despite the ability of the reciprocal experiment to work, could reflect the relative frequency of GA to GT (3:1) determinants available, or it could reflect an intrinsic effect on the B cell repertoire by the *Ir* gene, i.e., strain 13 guinea pigs could lack or not be able to use B cells with specificity for GA determinants.

Support for the latter hypothesis came from a careful study of the antibody response to DNP-oligolysine in guinea pigs. Levin *et al.* (1971) found that nonresponder (as defined by DTH) outbred guinea pigs could be primed with DNP-PLL if the adjuvant contained H37Rv *Mycobacterium* (10 mg/ml), which presumably served as a source of "schlepper" molecules. However, analysis by fluorescence quenching of the affinity of the antibodies produced revealed that responder and nonresponder guinea pigs differed in their ability to produce a high affinity antibody population specific for the immunogen. For example, immunization of responder guinea pigs with 14 ϵ-DNP-Lys_{14} in H37Rv containing CFA produced antibodies whose binding energy ($-\Delta F°$) could be proportioned into 70% for the DNP group and 30% for the carrier. If the carrier was varied in chain length from 2 to 18 it was clear that the maximum binding energy was achieved when the chain length was 14 ± 1. In contrast, a similar analysis of antibodies produced in nonresponder guinea pigs immunized with 14 ϵ-DNP-Lys_{14} in H37Rv containing CFA showed the same distribution of 70% of the binding energy for the hapten and 30% for the carrier, but no peak of carrier binding was found for a lysine chain length of 14. This result was interpreted as a direct influence of the *Ir* gene on which B cells could respond. The nonresponders appeared to lack or not use the higher affinity B cells specific for the immunizing antigen.

More recently, studies of protein antigens have confirmed these initial observations and rigorously shown that the phenomenon is controlled by MHC-linked *Ir* genes. Lozner *et al.* (1974) examined the antibody response to staphylococcal nuclease and found that B10.A mice were high responders after a single immunization in CFA, while B10 mice were low responders. However, after multiple immunizations Berzofsky *et al.* (1977) found that both strains produced the same total amount of antinuclease antibody. In looking for differences in the antibody responses of the two MHC-congenic

strains after hyperimmunization, the specificity of the antibody was assessed for separate parts of the molecule by measuring binding to radiolabeled fragments. In particular, it was found that B10.A mice had a significant portion of their antibodies directed against fragment 99–149 of the molecule, whereas the antibodies in B10 mice showed no detectable reaction with this fragment. In contrast, both strains made antibodies that bound to fragment 1–126. An analysis of the T cell proliferative responses in these two strains by R. Schwartz *et al.* (1978b) showed the same *Ir* gene control, i.e., T cells from B10.A mice immunized to whole nuclease, could be stimulated by fragments 99–149 or 1–126 but T cells from B10 mice could only be stimulated by fragment 1–126. All together, these results demonstrated that the determinant on fragment 1–126 could not serve as a helper determinant to facilitate the stimulation of B cells specific for fragment 99–149 in the low responder B10 strain. This suggests either that *Ir* genes directly influence which B cell clones can be stimulated or that a single carrier determinant can not "schlepp" for all B cell determinants on the molecule.

At the present time it is still not clear how *Ir* genes influence B cell specificity, even if only in some cases. Nonetheless, despite ignorance of the mechanisms involved, these observations focused attention on the fact that *Ir* genes did more than just influence T cell fine specificity and suggested the possibility that they might actually be expressed in B cells. Verification of this idea took a number of years to establish, as is outlined in the next few sections.

B. *Ir* Gene Expression in Lymphoid Tissue

1. Adoptive Transfer Experiments

The usual approach in immunology for demonstrating that a particular phenomenon is mediated by the immune system is to transfer lymphoid cells from animals of one phenotype to lethally irradiated animals of the other phenotype and show that the latter animals have now acquired the phenotype of the former. However, when McDevitt and Tyan (1968) first attempted such adoptive transfer experiments to show that the immune system was responsible for determining the Ir phenotype of the animals, they encountered an unanticipated problem. Whenever they transferred high responder spleen cells into low responder irradiated hosts, the animals would die from a graft-vs-host reaction, because they were invariably mismatched at the major histocompatibility complex. In contrast, the control experiments, i.e., the transfer of responder cells into irradiated responder hosts, almost always worked, because these animals turned out to be matched at the MHC. These observations presumably aided in the discovery of the linkage of *Ir* genes to the MHC, but they totally obscured the role of the immune system in mediating *Ir* gene function.

McDevitt and Tyan (1968) solved this problem by turning to F_1 crosses between high and low responder animals. These F_1 animals were responders to the antigen since responsiveness was dominant or codominant, yet they were tolerant to the MHC-encoded histocompatibility molecules of the low responder and, therefore, their cells could be used in an adoptive transfer experiment without causing a graft-vs-host reaction. When (C3H×C57BL/6)F_1 spleen cells from mice primed to (T,G)-A--L in CFA were transferred to irradiated (840 rads to eliminate host-vs-graft reactions) C3H low responder mice and boosted with (T,G)-A--L in saline at the time of transfer, or 10 days later, all but one of the recipients responded. The average antibody response to (T,G)-A--L was 56–59% (percentage of antigen bound in a Farr assay as described earlier, Section II,A), equivalent to that of primed and boosted normal F_1s (71%). Similar results were obtained when unprimed F_1 spleen cells were transferred into lethally irradiated C3H hosts and primed and boosted with (T,G)-A--L. In this case 4/16 (25%) of the animals did not respond. However, those which did respond produced antibody titers (52%) equivalent to irradiated F_1 recipients receiving F_1 spleen cells (63%) and significantly greater than irradiated C3H recipients receiving C3H spleen cells (8%). These results showed that responsiveness was an intrinsic property of the F_1 spleen cells and that radioresistant cells of the low responder mice did not influence the responsiveness of these mature lymphoid cells.

2. *Experiments with Limiting Dilution Analysis in Vivo*

In an attempt to determine whether a quantitative defect in antigen-specific lymphoid precursors (no distinction was made in the early experiments between T cells, B cells, and antigen-presenting cells) occurred in low responder mice, Mozes *et al.* (1970) used the adoptive transfer experiment under *in vivo* limiting dilution conditions to calculate precursor frequencies in high and low responder spleens. This was done by applying Poisson statistics to the number of irradiated recipients producing antibody 12–16 days later. For several branched chain synthetic polypeptide antigens they found that the precursor frequency for low responders was significantly lower (approximately 3 to 5-fold) than the precursor frequency for high responders. For example, the precursor frequency for the (T,G)-Pro--L response in high responder SJL (*H-2*^{s}) mice was 1 in 7.15×10^6. The precursor frequency for low responder DBA/1 (*H-2*^{q}) mice was 1 in 31.3×10^6. The 4.5-fold difference represented a minimal estimate for the difference in precursor frequencies since there was no evidence that the responses measured in the low responders resulted from qualitatively the same mechanism as was used by the responders, e.g., the low responders might produce antibody by a thymic-independent mechanism, whereas the high responders

might use a thymic-dependent mechanism [see earlier discussion of the (T,G)-A--L response in Section II,D].

In support of this concept were the results after priming with (T,G)-Pro--L. The SJL spleen cells manifested a 6-fold increase in precursor frequency after antigen priming (7.15×10^{-6} going to 1.26×10^{-6}). In contrast, the primed DBA/1 spleen cells were not significantly different from unprimed DBA/1 spleen cells (30×10^{-6} vs 31.3×10^{-6}). Thus, the primed SJL spleen cells displayed at least a 24-fold greater number of anti-(T,G)-Pro--L precursor cells than the DBA/1 spleen cells as a result of the expansion of the antigen-specific cells in the SJL in response to immunization. Clearly, the failure of the DBA/1 to manifest an expansion in precursors is part of the *Ir* gene defect. Again, however, the significance of the quantitative comparison is unclear since the DBA/1 precursors detected may represent a response mechanism used only in the low responder, because of the absence of the normal mechanism that operates in the high responder. If so, then the frequency comparison is only a minimum estimate of the difference.

One of the surprising aspects of these experiments was that the results conformed to the predictions from Poisson statistics for a single limiting cell type. The reason this is unexpected is because the antibody response depends on at least 3 cell types that have to interact: the antigen-presenting cell, the T cell, and the B cell. In order to have one limiting cell type, the other two must be present in much higher frequency (at least 10-fold). If the presenting cells are provided by the irradiated host in a large enough amount, then their number would remain constant throughout the titration and not influence the analysis. However, why both T and B cells should not have been limiting is unclear.

Mozes and Shearer (1971) made an attempt to distinguish which cell type was limiting by reconstituting the irradiated recipients with a mixture of bone marrow cells and thymocytes instead of spleen cells. One cell type was given in a fixed, large amount while the other was titrated in at limiting dilution. Surprisingly, they found that in most cases the bone marrow cells contained the 5-fold lower precursor frequency, similar to that found in the spleen of low responders. Limiting dilution curves for thymocytes showed no differences between high and low responders. In one case, the response to A--L containing branched chain copolymers, the thymocyte titrations did show a difference between high and low responders, but the data did not conform to the expected Poisson values for a single limiting cell type, so no quantitative assessment could be made.

These results demonstrated that the magnitude of the population of immunocompetent cells in the bone marrow provided a quantitative assessment of the *Ir* gene controlled difference between high and low responder mice. A number of investigators concluded that these results also demon-

strated that *Ir* genes function primarily in B cells, in contrast to all the data discussed earlier (Section II,D), which suggested T cells as the primary site of action. However, bone marrow is a complex mixture of cells and during extraction always becomes contaminated with peripheral blood cells from the vasculature of the bone. Thus, it is not clear in these experiments that the critical limiting cell type is a B cell (or B cell precursor). It could just as easily be an antigen-presenting cell (e.g., macrophage) precursor, which also derives from the marrow, or a mature T cell, since no attempt was made to remove contaminating peripheral blood T cells. Therefore, these studies did not conclusively show that *Ir* genes were expressed in B cells, although they did focus attention on the possibility that cells other than T cells might be involved.

C. *Ir* Gene Expression in Antigen-Presenting Cells

The possibility that an antigen-presenting cell, such as the macrophage, could be the site of expression of *Ir* genes seemed highly unlikely to many of the early investigators because of the way these genes appeared to influence the fine specificity of antigen recognition, a property felt to be solely that of a lymphocyte receptor. However, experiments by Green *et al.* (1967), which explored the transfer of DTH from responder to nonresponder guinea pigs, suggested that more than just lymphocytes might be involved. Lymph node cells from DNP-PLL or DNP-GL primed responder guinea pigs were transferred intravenously into nonimmune responder or nonresponder guinea pigs and the recipients skin tested 1 hour later. After 24 hours all responder recipients showed strong DTH reactions whereas almost none of the nonresponder recipients showed any DTH response. This failure of nonresponder recipients to manifest a DTH response to DNP-PLL or DNP-GL was not due to the rapid rejection of the donor cells because DTH to an antigen not under detectable *Ir* gene control, ovalbumin, was successfully transferred in ¾ of the cases (9 out of 12 guinea pigs). These results suggested that some host element was needed in addition to responder lymphocytes to elicit the DTH response and that this element also had to derive from a responder animal.

The demonstration that this host element was an antigen-presenting cell (macrophage) was first shown *in vitro* by Shevach and Rosenthal (1973). The basic design of the experiment was to measure the proliferative response of T lymphocytes from (responder × nonresponder)F_1 animals in the presence of macrophages from either the responder or nonresponder parental strain. The responsive F_1 T cells were tolerant to the histocompatibility antigens of both parents and therefore could respond to the antigen on either parental macrophage in the absence of any allogeneic effects caused by mixed lymphocyte reactions. In the system used by Shevach and Rosenthal, strain

(2×13)F_1 guinea pigs were immunized with DNP-GL (strain 2 is a responder, strain 13 is a nonresponder), GT (strain 13 is a responder, strain 2 is a nonresponder), and *Mycobacterium tuberculosis* (both strains 2 and 13 are responders). T cells were purified from peritoneal exudates by passage over rayon wool columns and stimulated in culture with nonimmune, mitomycin C-inactivated peritoneal macrophages containing one of the three antigens on their surface (pulsed macrophages). Macrophages from F_1 or responder guinea pigs presented antigen to primed F_1 T cells, but macrophages from nonresponder animals did not. For example, strain 2 macrophages could present DNP-GL but not GT, whereas strain 13 macrophages could present GT but not DNP-GL. F_1 macrophages could present both DNP-GL and GT and all three macrophages could present purified protein derivative of tuberculin (PPD), an antigen to which all three strains respond. It was concluded from these experiments that nonresponder macrophages selectively fail to present antigens to which they are nonresponders. This implied that the *Ir* genes were expressed in the macrophage, a cell type that controls and regulates T cell activation, rather than in the T lymphocytes themselves.

This seminal observation was confirmed in the mouse by Yano *et al.* (1978) who demonstrated that *Ir* genes linked to the MHC were responsible for the effect. B10.A (H-2^a) mice were high responders to the random terpolymer GAT while B10.Q (H-2^q) mice were nonresponders. T cells from the peritoneal cavity of (B10.A × B10.Q)F_1 responder mice primed with GAT could respond to GAT-pulsed F_1 or high responder antigen-presenting cells (irradiated spleen cells) but not to GAT-pulsed low responder antigen-presenting cells. Mixing of high and low responder antigen-presenting cells did not inhibit the presentation of GAT by the high responder macrophages. In addition, treatment of the responding population with anti-Lyt 2 and complement prior to stimulation with GAT-pulsed low responder macrophages did not unmask a T cell proliferative response. These last two experiments argued against the idea that the failure to respond to GAT on low responder macrophages was due to a suppressor T cell mechanism operating at the time of *in vitro* stimulation. Thus, low responder macrophages appeared to be intrinsically defective in presenting GAT to primed F_1 responder T cells.

One could argue that a secondary T cell proliferative response was too late an assay system to detect suppressor T cells, which may operate only during the initial priming of the animal. As we will discuss later (Section VI,V,4), this may be the case in the GAT system; however, a number of other investigators have examined primary responses, both proliferative (Thomas, 1978) and helper (Singer *et al.*, 1978), and obtained results identical to those described above. For example, Singer *et al.* (1978) examined the primary plaque-forming cell response to trinitrophenyl (TNP)-conjugated (T,G)-A--L and (H,G)-A--L by (B10 × B10.A)F_1 spleen cells. B10 (H-2^b) mice were high

responders to (T,G)-A--L and low responders to (H,G)-A--L, whereas B10.A (*H-2*^{a}) mice were just the opposite. Derivatization of the antigens with TNP did not alter this MHC-linked *Ir* gene control. When unprimed (B10 × B10.A)F_1 spleen cells were depleted of antigen-presenting cells by passage over Sephadex G-10, they failed to respond to either TNP-(T,G)-A--L or TNP-(H,G)-A--L. Addition of F_1 splenic adherent cells (SAC) as a source of antigen-presenting cells reconstituted the response to both antigens. In contrast, addition of B10 SAC only reconstituted the response to TNP-(T,G)-A--L, and addition of B10.A SAC only reconstituted the response to TNP-(H,G)-A--L. B10.A(4R) (K^k,*I-A*^{k},*I-E*^{b},D^b) SAC behaved like B10.A SAC and B10.A(5R) (K^b,*I-A*^{b},*I-E*$^{b/a}$,D^d) SAC behaved like B10 SAC. These results with recombinant strains mapped the regulation of antigen presentation to the *K* region or *I-A* subregion of the MHC, identical to the original mapping of the *Ir* genes by McDevitt *et al.* (1972) based on antibody formation in the whole animal. Finally, a mixture of B10.A(4R) and B10.A(5R) SACs was capable of presenting both antigens, demonstrating that the failure of each SAC population to present one of the two antigens was not the result of the induction of an antigen-specific suppression mechanism. These results demonstrated that, even in a primary response, *Ir* genes manifest themselves in antigen-presenting cell function.

The DTH transfer results mentioned earlier can now be understood in terms of the requirement for high responder macrophages to elicit a T cell response. This was shown by Miller *et al.* (1977) who transferred GAT-primed T cells from (responder × nonresponder)F_1 mice into either naive responder F_1, parental responder (BALB/c or A.TL), or parental nonresponder (SJL or A.TH) mice, and tested for DTH by measuring the uptake of 5-[^{125}I]iodo-2′-deoxyuridine into the site of antigen challenge. F_1 and parental responder recipients supported a DTH response, whereas nonresponder parental recipients did not. These results were interpreted to mean that the dendritic-like cells in the skin (Langerhan's cells) were only able to elicit a DTH response from the transferred T cells if the Langerhans' cells were of responder genotype, similar to the interpretation given to the *in vitro* T cell proliferation results. Thus, an overwhelming body of evidence suggested that *Ir* genes were expressed in antigen-presenting cells.

D. *Ir* Gene Expression in B Cells

The experimental design used to demonstrate *Ir* gene expression in B cells was identical to that used for macrophages. This was first carried out *in vivo* by Katz *et al.* (1973). T cells from (responder × nonresponder)F_1 mice [BALB/c (*H-2*^{d}) × A/J (*H-2*^{a})] were primed with the antigen, GLT, or as a control, KLH, and then transferred into an unprimed F_1 host. Twenty-four hours later the animals were irradiated with 600 R, injected with "B cells"

(anti-Thy 1 and complement treated spleen cells) previously primed to DNP-KLH, and reimmunized with either DNP-KLH or DNP-GLT. Anti-DNP antibodies were measured in the serum 7 days later. F_1 T cells could cooperate with either parental B cell population to produce anti-DNP antibody when DNP-KLH was used as the antigen. In contrast, when DNP-GLT was used as the antigen, only high responder BALB/c B cells functioned. The failure of the low responder A/J B cells to function could have resulted from the failure to activate the T cells with responder macrophages since, as described in Section III,C, these are required for T cell activation. It is not clear whether the antigen-presenting cells in this helper T cell model are provided by the responder F_1 irradiated host or the nonresponder macrophages in the donated B cell population. To circumvent this problem, another experimental group was added to which the antigen was introduced in the form of DNP-GLT pulsed F_1 peritoneal macrophages (Katz, 1977a). This form of the antigen was still sufficient to stimulate an anti-DNP-antibody response in high responder B cells but was insufficient to stimulate a response in the low responder B cell population. These results suggested that *Ir* genes were expressed in B cells.

Confirmation of these findings *in vitro* was achieved by Marrack and Kappler (1978). In their system (high responder × low responder) (B6×A)F_1 T cells were primed with (T,G)-A--L *in vivo*, purified by passage over nylon wool columns, and restimulated *in vitro* with TNP-(T,G)-A--L-pulsed peritoneal washout cells as a source of antigen-presenting cells (macrophages). B cells were prepared from TNP-lipopolysaccharide (LPS)-primed spleen cells by removal of T cells with anti-Thy 1 and complement and removal of macrophages by passage over Sephadex G-10. (B6×A)F_1 T cells helped high responder B10 B cells in the presence of B10 macrophages. In the presence of B10.A macrophages, however, no help was observed, demonstrating the requirement for high responder antigen-presenting cells. When low responder B10.A B cells were used, no anti-TNP PFC responses were observed in the presence of either high responder or low responder macrophages. The failure of high responder macrophages and T cells to stimulate a response from low responder B cells demonstrated that the B cells must also be of high responder genotype in order to generate an antibody response. Thus, it appeared from a direct study of sites of *Ir* gene expression that both the B cell and the antigen-presenting cell were cell types that expressed these genes.

E. *Ir* Gene Expression in T Cells

1. *Experiments with Radiation-Induced Bone Marrow Chimeras*

If one reviews the early evidence that suggested that *Ir* genes were expressed in T cells (Section II,D), it will be noted that all of it was indirect. The experiments showed that T cell function was involved, but did not

localize the site of *Ir* gene expression to the T cell itself. In many of those experiments the antigen-presenting cell could have been the critical cell type determining responsiveness. Thus, in the late 1970s, several laboratories began to ask again whether *Ir* genes were expressed in T cells.

This was a more difficult question to ask for T cells than it was for B cells and antigen-presenting cells, because simple mixing experiments of high and low responder T cells with F_1 B cells and macrophages were complicated by T cell alloreactions against the MHC-encoded antigens of the other parent. This problem was circumvented through the use of radiation-induced bone marrow chimeras (see Longo *et al.*, 1981, for a review). T cell-depleted bone marrow or fetal liver cells were injected into lethally irradiated (850 to 1000 R) recipients and allowed 2–3 months to reconstitute the animal's immune system. As a result of this manipulation, the chimeric T cells were tolerant to host as well as donor MHC-encoded antigens. This made it possible to mix cells from responder and nonresponder mice without any complicating allogeneic reactions.

2. *Helper T Cells and the (T,G)-A- -L Response*

In the first such study, Kappler and Marrack (1978) set up the following radiation-induced bone marrow chimera to examine the (T,G)-A--L response: T cell-depleted bone marrow from both the high responder B10 and the low responder B10.A strains were injected into lethally irradiated $(B6\times A)F_1$ recipients. The abbreviated designation for these chimeras is B10(b.m.) + B10.A(b.m.) → $(B6\times A)F_1$(irr). Eight weeks later the chimeras were immunized and the draining lymph nodes used 7 days after priming as a source of helper T cells. Note that because the chimera had been reconstituted with bone marrow from both strains, the priming environment contained both high and low responder antigen-presenting cells and thus should not be limiting in any way. The helper T cell population was treated with anti-$H\text{-}2^b$ antisera and complement to remove the B10 high responder T cells, and the remaining chimeric B10.A T cells tested for helper activity with TNP-(T,G)-A--L and B cells and macrophages (anti-Thy 1 and complement treated spleen cells) from TNP-LPS primed high responder B10 mice. The genotypic low responder B10.A T cells provided as much helper activity as normal $(B6\times A)F_1$ T cells. Similar experiments were performed with single parent into F_1 chimeras: B10.A(b.m.) → $(B6\times A)F_1$(irr). In this case 10^8 anti-Thy 1 and complement treated F_1 spleen cells were injected into the chimeras at the time of priming with (T,G)-A--L in order to provide a source of high responder antigen-presenting cells. Again, the chimeric B10.A T cells were able to help high responder B cells and macrophages produce anti-TNP PFC to TNP-(T,G)-A--L. In contrast, these T cells could not help TNP-LPS primed low responder B10.A macrophages and B cells when (T,G)-A--L was the carrier, although B10.A chimeric T cells functioned quite

well when KLH was the carrier. Thus, a genotypic low responder T cell population, which had matured in a high responder F_1 environment, behaved as a phenotypic responder, i.e., the *Ir* gene was not functioning in the T cell. At the same time, the failure to help low responder B10.A macrophages and B cells supported the previous conclusion that these two cell types express *Ir* gene products.

In order to determine whether the low responder T cell population normally possessed cells capable of helping high responder B cells and macrophages to respond to TNP-(T,G)-A--L, or whether the environment in which the T cell population developed played a critical role, Kappler and Marrack also constructed chimeras of the F_1(b.m.) → Parent(irr) type. T cells from (B6×A)F_1(b.m.) → B10(irr) high responder chimeras were capable of helping B10 high responder B cells and macrophages respond to TNP-(T,G)-A--L, whereas T cells from (B6×A)F_1(b.m.) → B10.A(irr) low responder chimeras were not. T cells from neither chimera would help low responder B10.A B cells and macrophages. The failure of (B6×A)F_1(b.m.) → B10.A(irr) T cells to help high responder B10 B cells and macrophages was not due to the presence of antigen-specific suppressor cells, because mixing these T cells with T cells from (B6×A)F_1(b.m.) → B10(irr) chimeras did not prevent the latter from helping B10 B cells and macrophages. These results demonstrated that the ability of the high responder (B6×A)F_1 T cells to help B10 high responder B cells and macrophages was an acquired characteristic. When the T cells developed in a high responder environment (B10) they contained such helper cells; when the T cells developed in a low responder environment (B10.A) they did not contain such helper cells. Thus, tolerance alone was not sufficient to unmask these helper T cells. The presence of high responder alleles in the irradiated host during T cell development was essential. These results suggested that in B10.A(b.m.) → (B6×A)F_1(irr) chimeras the T cell repertoire was actually expanded during development to include helper cells specific for TNP-(T,G)-A--L in association with high responder B cells and macrophages. Note again that under no circumstances did any chimeric T cell population acquire or manifest the ability to help low responder B10.A B cells and macrophages.

3. T Cells Mediating DTH and the GAT Response

At about the same time as Kappler and Marrack, Miller *et al.* (1979) demonstrated essentially the same results for T cells mediating delayed-type hypersensitivity (DTH). Using GAT as the antigen, A.SW (*H*-2^s) as the nonresponder strain and BALB/c (*H*-2^d) as the responder strain, Miller set up chimeras in which the recipients were adult thymectomized and given a thymus graft before irradiation (split dose: 500 rads followed 2 weeks later by 700 rads) and reconstitution with T cell-depleted bone marrow. Chimeras of

the parent into F_1 type are designated P_1(b.m.) + F_1(Thy) → F_1(ATX) and chimeras of the F_1 into parent type are designated F_1(b.m.) + P_1(Thy) → F_1(ATX). Responder BALB(b.m.) + responder (BALB×A.SW)F_1(Thy) → responder (BALB×A.SW)F_1(ATX) chimeric T cells could mount a DTH response against GAT when immunized with the antigen in complete Freund's adjuvant. In contrast, nonresponder A.SW(b.m.) + responder (BALB×A.SW)F_1(Thy) → responder (BALB×A.SW)F_1(ATX) chimeric T cells could not. However, when the latter chimeric animals were immunized and sensitized with (BALB × A.SW)F_1 peritoneal exudate macrophages pulsed with GAT, to provide a source of responder antigen-presenting cells that are missing in these chimeras, they now responded as well as the former chimeric animals to GAT. Thus, nonresponder T cells that had matured in a responder environment were capable of responding to the antigen in association with responder macrophages, but not in association with nonresponder macrophages.

This acquisition of responsiveness by nonresponder T cells was paralleled by a loss of responsiveness in responder (BALB × A.SW)F_1(b.m.) + nonresponder A.SW(Thy) → responder (BALB × A.SW)F_1(ATX) chimeras. These animals gave a barely detectable response to GAT and could not transfer DTH to naive responder BALB/c or nonresponder A.SW recipients. This was in contrast to the responder (BALB/c × A.SW)F_1(b.m.) + responder BALB(Thy) → responder (BALB × A.SW)F_1(ATX) chimeras, which responded to GAT and could transfer DTH to a responder BALB/c recipient. Thus, the genotypic responder T cell population behaved as a nonresponder population when it matured in a nonresponder environment. Note that the only environmental component that came from the nonresponder in this experiment was the thymus.

4. The Role of the Thymus in Determining Ir Phenotype

Confirmation that the thymus was the critical organ that determined the Ir phenotype of the T cells was obtained with a T cell helper assay for calf skin collagen by Hedrick and Watson (1979). C57BL/6 (H-2^b) mice are high responders to collagen while BALB/c (H-2^d) mice are low responders. When (BALB × C57BL/6)F_1 nude mice were grafted with a thymus from either parent and immunized with collagen 3 months later, the B6(Thy) → (BALB × B6)F_1(Nu) chimeras produced high antibody titers while the BALB/c(Thy) → (BALB × B6)F_1(Nu) chimeras produced low antibody titers. Antibodies to sheep red blood cells, after immunization with this antigen, were comparable in the two sets of chimeras. Thus, in both the nude and radiation-induced bone marrow chimera models, the thymus was clearly established as a site which could directly or indirectly influence the acquisition of the Ir phenotype of the T cell repertoire.

5. *Allogeneic Chimeras*

Extension of the concept of the acquired nature of the T cell Ir phenotype to an allogeneic chimera model was first reported by Singer *et al.* (1981). Using TNP-(T,G)-A--L as the antigen, B10 mice (H-2^b) as the high responder strain, and B10.A mice (H-2^a) as the low responder strain, they set up both types of allogeneic chimeras, B10(b.m.) → B10.A(irr) and B10.A(b.m.) → B10(irr). Spleen cells were removed from the chimeras 3 months after reconstitution and passed over Sephadex G-10 to eliminate macrophages. The remaining lymphocytes were supplemented with (B10 × B10.A)F_1 splenic adherent cells (SAC), as a source of responder antigen-presenting cells, and stimulated with TNP-KLH or TNP-(T,G)-A--L in a primary anti-TNP antibody assay. Both allogeneic chimeras responded to TNP-KLH in the presence of F_1 SAC, demonstrating the functional integrity of the cells. However, in the presence of TNP-(T,G)-A--L only the B10.A(b.m.) → B10(irr) chimeric cells responded, and they did so as well as the F_1 control cells. Lymphocytes from the high responder B10(b.m.) → low responder B10.A(irr) chimera did not respond at all. Thus, there was a complete reversal of the normal Ir phenotype. High responder B10 lymphocytes behaved as low responders and low responder B10.A lymphocytes behaved as high responders. The developmental environment of the host completely determined the Ir phenotype.

6. *Allophenic Mice*

Finally, Erb *et al.* (1980) demonstrated similar results in allophenic chimeras (designated A ↔ B). This unique model, developed by Mintz (1971), involves fusing allogeneic mouse embryos at the 8 cell stage and implanting the aggregated blastocysts into pseudopregnant females. The resulting offspring are usually mosaics in their lymphoid system and tolerant to skin grafts from either parental donor. Erb *et al.* set up C57 ↔ BALB/c allophenic chimeras between C57BL/6 (H-2^b), which is a nonresponder to pork insulin, and BALB/c (H-2^d), which is a responder to this antigen. Nylon wool-purified T cells from a pool of 4 allophenics were H-2 typed as being 82% H-2^b-bearing cells and 17% H-2^d-bearing cells. The H-2^d-bearing cells were removed by treating the population with anti-H-2^d antibodies and complement and the remaining H-2^b-bearing cells were examined for helper activity in a two stage *in vitro* assay. The T cells were incubated with pork insulin in the presence of high responder BALB/c or low responder C57 macrophages for 4 days and then tested for helper activity in a second assay with (B10×B10.D2)F_1 B cells and macrophages in the presence of DNP-pork insulin. The low responder genotype, H-2^b-bearing T cells responded to pork insulin when it was presented on high responder BALB/c macrophages, but not when the antigen was presented on low responder C57 macrophages. Thus, in line with all the previous examples, the low re-

TABLE II
THE T CELL Ir PHENOTYPE IS AN ACQUIRED PROPERTY LEARNED DURING DEVELOPMENT, WHEREAS THE B CELL AND ANTIGEN-PRESENTING CELL Ir PHENOTYPES ARE GERMLINE ENCODED

Stem cell Ir genotype	Thymic Ir genotype	Immune response when B cells and APCs[a] come from	
		High responder	Low responder
Low	(High × low)F_1	High	Low
Low	High	High	Low
(High × low)F_1	Low	Low	Low
High	Low	Low	Low
(High × low)F_1	High	High	Low
High	(High × low)F_1	High	Low

[a] APCs, antigen-presenting cells.

sponder T cell population from the allophenic chimera appeared to have acquired the Ir phenotype of the high responder.

7. *Summary*

A schematic summary of these chimera experiments is presented in Table II.

The major conclusion to emerge from all of these experiments is that the T cell Ir phenotype is an acquired characteristic. The T cell population, regardless of its genotype, adopts the Ir characteristics of the MHC haplotype of the thymus in which it develops. The nature of the learning process is not clear, but it must involve interaction with a relatively radioresistant cell in the thymus. In all cases so far examined, the T cell population has only been able to gain or lose the ability to respond to the antigen in association with high responder macrophages and B cells. The inability to respond to antigen in association with low responder macrophages and B cells remains unaltered. From these observations one can conclude that *Ir* genes are expressed in B cells and macrophages, but not in T cells.

IV. Parallels between MHC Restriction and *Ir* Gene Control

Striking parallels exist between the phenomena of MHC restriction and *Ir* gene control.[1] An almost perfect correlation exists in the chimera studies in

[1] MHC restriction is the phenomenology which stems from the requirement of T cells to corecognize antigen and a particular allelic form of a gene product of the MHC in order to become activated. If the reader is not familiar with the phenomenon of MHC restriction, he or she is referred to an extensive chapter on the topic published in the textbook "Fundamental Immunology" (Schwartz, 1984).

terms of what happens to the Ir phenotype and what happens to the T cell restriction specificity. For example, P_A(b.m.) → (A×C)F_1(irr) chimeric T cells have been shown to possess an expanded repertoire in that they can recognize antigen in association with MHC-encoded molecules from either parent C or parent A. If parent A was a low responder to a particular antigen and parent C a high responder, then the P_A(b.m.) → (A×C)F_1(irr) chimeric T cells were shown to be high responders. However, this meant responding only with high responder strain C macrophages and B cells. Thus, the acquisition of responsiveness appeared to result from the ability to recognize responder MHC-encoded gene products as self restricting elements and not from acquiring an ability to respond to the antigen in association with low responder B cells and macrophages.

In the (A×C)F_1(b.m.) → P_A(irr) chimeras, the T cell population was shown to possess a contracted repertoire in that the cells could only recognize antigen in association with parent A MHC-encoded gene products. If parent A was a low responder to the antigen, then the T cell population was nonresponsive. In this case the loss of responsiveness by the genotypic F_1 responder T cells meant the loss of the ability to recognize high responder strain C MHC-encoded gene products. This result implies that the normal strain A T cell repertoire does not contain clones that recognize the antigen in association with high responder strain C B cells and macrophages, because if it did, the introduction of (A×C)F_1 antigen-presenting cells in the chimera would result in a responder phenotype. The only (and perhaps critical) exception to this analysis is if the clones in strain A that recognize the antigen in association with strain C MHC-encoded molecules are part of the alloreactive pool directed against strain C MHC-encoded molecules alone. In this case, such clones would be deleted by a tolerance mechanism and not be detected. As a corollary, such clones would necessarily be different from the antigen-specific clones normally found in the high responder strain C, as the alloreactive clones should also be deleted by a tolerance mechanism in the strain C animal.

The allogeneic chimeras showed both the gain and loss characteristics of the two types of semiallogeneic chimeras, combined into one. T cells from A(b.m.) → C(irr) chimeras generally showed a gain in the ability to recognize antigen in association with strain C MHC-encoded gene products and a loss of the ability to recognize antigen in association with strain A MHC-encoded gene products. If strain A was a nonresponder and strain C a responder to the antigen, then A(b.m.) → C(irr) chimeric cells gained the ability to respond to the antigen in association with strain C macrophages and B cells, while T cells from the reciprocal allogeneic chimera showed a loss of this responder capacity. All of these results show that Ir phenotype is correlated with and possibly dependent upon the MHC restriction specificity of the animal's T cells. Both phenomena appear to be related to the T cell recogni-

tion capacity of the animal, an acquired characteristic that depends on the MHC-encoded molecules expressed in the thymus during T cell development. Thus, the possibility was raised that the same MHC-encoded molecules mediate both functional attributes.

The genes that code for Ia molecules, the structural products that function as restriction elements for helper, DTH and proliferative T cells, were localized to the same genetic region of the MHC as the *Ir* genes. In addition, the Ia molecules were predominantly found on B cells and macrophages and not on resting T lymphocytes. This correlated with the expression of *Ir* genes, which manifested themselves in B cells and macrophages, but not in T cells. Thus, as first pointed out by Shevach and Rosenthal (1973) and Katz *et al.* (1973), the Ia molecules involved in cellular interactions may in fact be the *Ir* gene products or be closely regulated by such products.

The clearest way to visualize the parallel relationship between Ia molecules and *Ir* gene products is to consider the lymphocyte composition of the F_1 animal (Fig. 1). In the normal (A×C)F_1 response to a conventional anti-

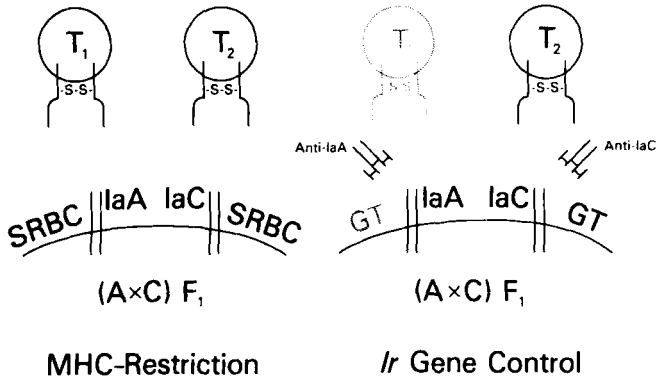


FIG. 1. Parallels between the immunological phenomena of MHC restriction and *Ir* gene control. In an F_1 animal (A×C), usually two different subpopulations of T cells exist (T_1 and T_2) specific for the same antigen (sheep red blood cells, SRBC). T_1 recognizes the SRBC in association with an Ia molecule encoded by a gene from the A haplotype (IaA) and T_2 recognizes the SRBC in association with a different Ia molecule encoded by a gene from the C haplotype (IaC). In contrast, for antigens, the response to which is controlled by an *Ir* gene, e.g., poly$(Glu^{50},Tyr^{50})_n$ (GT), only one subpopulation of T cells appears to respond, the one (T_2) that recognizes the antigen in association with an Ia molecule (IaC) encoded by the responder MHC haplotype (C). The T cell subpopulation (T_1) that would be expected to normally respond to the antigen in association with an Ia molecule (IaA) encoded by the nonresponder MHC haplotype (A) either does not exist (dotted T_1 cell), or, if it does exist, cannot be stimulated, possibly because the GT molecule cannot associate with the IaA molecule (dotted GT). As a consequence of this biological set up, antibodies directed against the IaC molecule will inhibit the response to GT, whereas antibodies directed against the IaA molecule will not, because no T_1 cells with specificity for the IaA molecule participate in the response.

gen, such as sheep red blood cells (SRBC), two subpopulations of T lymphocytes can be discerned. One is specific for SRBC in association with strain A MHC-encoded Ia molecules; the other is specific for SRBC in association with strain C Ia molecules. These T cells have a receptor or receptors that initially recognize the antigen in association with Ia molecules on the surface of an antigen-presenting cell (macrophages). If the T cell is a helper cell, then the same T cell in turn can react with antigen on the surface of a B cell, which also expresses Ia molecules.

Now let us consider the special case of an antigen, the response to which is under *Ir* gene control (Fig. 1). In this case the $(A \times C)F_1$ represents a cross between the high responder strain C and the low responder Strain A. When this animal is immunized, it responds to the antigen, but an analysis of the specificity of the response shows that only T cells specific for antigen in association with the high responder strain C macrophages and B cells are detected. Recall that low responder strain A macrophages and B cells do not appear to function with the antigen. This can be interpreted as a failure to stimulate the T cell subpopulation with specificity for the antigen in association with the strain A Ia molecule, or as the absence of T cell clones with that specificity (dotted lines in Fig. 1). But whatever the mechanism for the nonresponsiveness, it can be seen that the *Ir* gene regulation is just a special case of MHC-restricted T cell recognition in which a subpopulation of T cells with specificity for the antigen in association with a particular Ia molecule is not detected.

A clear experimental example that can be interpreted in this theoretical framework comes from the studies of Barcinski and Rosenthal (1977) on the genetic control of the immune response to insulin in inbred guinea pigs. Both strain 2 and strain 13 guinea pigs respond to pork insulin when immunized with the antigen in CFA. However, a careful analysis of the antigen specificity of the response, done by comparing cross-reactivity patterns for insulins from different species, revealed that strain 2 guinea pigs responded to a determinant involving residues A8 to A10 on the A chain disulfide loop whereas strain 13 guinea pigs responded to His-10 on the B chain. When $(2 \times 13)F_1$ guinea pigs were immunized with insulin and their T cells challenged *in vitro* with insulin-pulsed strain 2 or strain 13 macrophages, both parental macrophages induced a T cell proliferative response. Using a bromodeoxyuridine and light elimination protocol, Rosenthal *et al.* (1977) demonstrated that different subpopulations of F_1 T cells responded to insulin in association with each of the parental macrophages. Thus, under these conditions, the response appeared to be similar to a normal F_1 response in which two subpopulations of T cells could be detected, one specific for the antigen in association with each of the two MHC-encoded restriction elements (Ia molecules). However, when isolated B chain was used as the immunizing

antigen in these F_1 experiments, only strain 13 macrophages presented the antigen. Strain 2 macrophages failed to present the B chain, presumably because the T cell clone that recognized the His-10 on the B chain was specific for the strain 13 Ia molecule. If there had been a clone primed in the F_1 that was specific for the B chain in association with the strain 2 Ia molecule, then strain 2 macrophages would have presented the B chain to it. The failure to detect such clones in the F_1 animal is of course an *Ir* gene regulated phenomenon. Strain 2 guinea pigs are nonresponders to the B chain of insulin whereas strain 13 guinea pigs are responders. Thus, when an individual antigen is examined at the determinant level it is clear that *Ir* gene control is at least in part a consequence of the MHC-restricted recognition by the T cells. In those cases in which the antigenic complexity is reduced sufficiently to allow detection of this determinant-specific, restricted recognition simply by immunization, the antigen will be classified in the special category of being under *Ir* gene control. In those cases in which the antigen is more complex, i.e., has a greater number of detectable determinants, the antigen will appear not to be under *Ir* gene control.

This analysis, of course, depends completely on the assumption that the Ia molecules, which serve as restriction elements for the T cells, are either the *Ir* gene products themselves or are regulated in their function by such products. The data presented so far demonstrate only that closely linked genes expressed in similar cell types are involved in both phenomena. In the next section we will present more extensive experimental data that strengthen the correlation between *Ir* gene products and Ia molecules and the most recent data on *I* region mutations that prove that the two molecules are in fact identical.

V. Ia Molecules Are the *Ir* Gene Products

A. Antibodies against Ia Molecules

1. The Discovery of Ia Molecules

The initial discovery of the *I* region as the genetic location of *Ir* genes led several investigators to attempt to identify the products of these genes by serological techniques (reviewed in Shreffler and David, 1975). Using recombinant strains of mice that differed mainly in this genetic region of the MHC, such as A.TL ($K^sI^kS^kD^d$) and A.TH ($K^sI^sS^sD^d$), they immunized one of these strains with spleen and lymph node cells from the other. After hyperimmunization (6–8 injections) sera from the animals were obtained and tested for their reactivity against various cells in the immune system. Because the major theory at the time suggested that *Ir* genes might encode the

T cell receptor, most investigators expected that their antisera would react with T lymphocytes. Surprisingly, most of these antisera reacted with B cells and macrophages (Sachs and Cone, 1973), although some rare sera did react with T lymphocytes (Götze, 1978). Because of this unexpected result, the workers in the field hesitated to call the molecules being detected by these antisera *Ir* gene products and instead termed them *I* region-associated or Ia molecules to indicate the genetic linkage. More recently these molecules have been called MHC-encoded class II molecules. The chemistry of these molecules (reviewed by Strominger *et al.*, 1981; Kindt and Robinson, 1983; Kaufman *et al.*, 1984) revealed a two chain structure, α (33–35K) and β (27–29K), both of which are transmembrane glycoproteins found noncovalently associated on the cell surface. Each chain is composed of two extracellular domains, with the N-terminal (α_1 and β_1) domains containing the majority of the structural polymorphism found to exist between different allelic forms of the molecules. In the mouse, two such protein pairs exist, A_β:A_α and E_β:E_α, although not all strains express an E_β:E_α molecule.

2. *Inhibition of T Cell Proliferation*

The first evidence to suggest a functional role for Ia molecules in an immune response came from studies demonstrating the *in vitro* inhibition of a T cell proliferation assay using anti-Ia antisera. Antisera containing antibodies directed against Ia molecules completely inhibited the T cell proliferative response to a variety of antigens (although not all of them) when added in small amounts (1–2% v/v) for the duration of the culture period. For example, an A.TH anti-A.TL antiserum (I^s anti-I^k) blocked the proliferative response of B10.A ($I^{k/d}$) T cells to the IgA myeloma protein, TEPC-15, the B10.BR (I^k) response to (Phe,G)-A--L, and the B10.D2 (I^d) response to GLT, but not the B10 (I^b) response to (T,G)-A--L (R. Schwartz *et al.*, 1976a). The inhibition of B10.D2 cells was through a serologic cross-reaction of anti-I^k on I^d (referred to as a public specificity). In general, blocking required that at least one cell type in the responding population bear an Ia determinant recognized by the antisera and that the titer of the relevant anti-Ia antibodies be fairly high. Antisera containing antibodies directed against *H-2K* and *D*-encoded molecules (class I molecules) did not significantly inhibit the response, e.g., A.TL anti-A.AL for B10.A T cell responses.

More recently, monoclonal anti-Ia antibodies have been shown to have similar effects, thus proving that antibodies reacting with Ia molecules and not other contaminating antibodies in the whole sera were responsible for the inhibition (for a review see Nagy *et al.*, 1981). In addition, these experiments clearly demonstrated that individual monoclonal antibodies could be highly selective in their inhibition of responses involving individual Ia mole-

cules. For example, Lerner *et al.* (1980) demonstrated that the monoclonal antibody, Y-17 (Ia.m44), which reacts with Ia molecules encoded in the *I-E* subregion (E_β:E_α), inhibited the T cell proliferative response to pigeon cytochrome *c* and the terpolymer, GLPhe, but did not inhibit the response to (T,G)-A- -L. In the reciprocal direction, Baxevanis *et al.* (1980) showed that the monoclonal antibody, Ia.m2, which reacts with Ia molecules encoded in the *I-A* subregion (A_β:A_α), inhibited the T cell proliferative response to the copolymer GA but did not inhibit the response to the terpolymer, GLT. Similar selectivity in monoclonal blocking was reported by Nepom *et al.* (1981).

The most interesting result with monoclonals, however, has been the demonstration that an antibody reacting with a single Ia molecule could inhibit the response to one but not another of two antigens, both utilizing the same Ia molecule as a restriction element. This was first shown by Burger and Shevach (1980) in the guinea pig, using xenogeneic mouse anti-guinea pig Ia molecule monoclonals. One monoclonal, 27E7, inhibited the T cell proliferative response to PPD and the GL copolymer as strongly as a strain 13 anti-strain 2 anti-Ia alloantiserum. In contrast, monoclonal 22C4 inhibited the PPD response to the same extent as the other reagents but did not significantly inhibit the response to GL. Biochemical studies involving sequential immunoprecipitation demonstrated that the two monoclonals, 27E7 and 22C4, reacted with the same Ia molecule. Similar experiments were performed in the mouse by Needleman *et al.* (1984). These results suggest that different monoclonal antibodies react with different parts of the Ia molecule and that these parts (determinants) serve different functional roles in antigen presentation, i.e., some antigens are recognized in association with one Ia determinant, some in association with another. However, a note of caution has recently been introduced by Beck *et al.* (1983) in relying too heavily on the mapping of Ia determinants on the molecule with anti-Ia monoclonals. Their results suggest that sometimes the blocking is steric and thus affects sites distant from the determinant recognized by the monoclonal antibody.

3. *Blocking Occurs at the Level of the Antigen-Presenting Cell*

Knowing that the anti-Ia antisera inhibit T cell proliferative responses by reacting with Ia molecules suggested that the antisera were blocking antigen-presenting cell function, since Ia molecules are easily detected on these cells and not detectable on most T lymphocytes. However, some T lymphocytes have been reported to express Ia molecules and the possibility existed that the failure to detect these molecules on all T lymphocytes was a problem in sensitivity of the assay systems. In order to unequivocally establish the cellular site of action of anti-Ia antibodies it was necessary to turn to the use

of radiation-induced bone marrow chimeras to create a situation in which the T cells and macrophages expressed different MHC-encoded molecules (Hodes *et al.*, 1980; Longo and Schwartz, 1981). In the experiments of Longo and Schwartz (1981) chimeric B10.A T cells that had matured in a (B10.A×B10.Q)F_1 environment acquired the ability to respond to GLPhe, an antigen to which the B10.A mouse is normally a nonresponder. The response of these chimeric B10.A T cells was initiated by GLPhe in association with responder B10.Q antigen-presenting cells (APCs) but not by GLPhe in association with nonresponder B10.A APCs. Addition of an anti-H-2^q antiserum containing anti-Ia^q antibodies directed against the B10.Q APCs (but not the B10.A T cells) inhibited the GLPhe response. In contrast, both anti-Ia^k antisera and a monoclonal anti-Ia^k antibody (Ia.m27), specific for gene products of the MHC haplotype of the responding B10.A T cells (but not the stimulating B10.Q APCs) did not substantially inhibit the GLPhe response. The monoclonal antibody and the anti-Ia^k antisera were functional since they could completely inhibit the response of the B10.A T cells to another antigen, GAT, which requires B10.A-presenting cells (Ia^k) as a source of high responder APCs. In the reciprocal experiment, chimeric B10.T(6R) T cells (I^q) that had matured in a (B10.A×B10.Q)F_1 environment acquired the ability to respond to GAT, an antigen to which the B10.T(6R) mouse is normally a nonresponder. The response of the chimeric B10.T(6R) T cells was initiated by GAT in association with responder B10.A APCs but not by GAT in association with nonresponder B10.T(6R) APCs. Addition of the monoclonal Ia.m27 or anti-Ia^k antisera, with specificity for Ia molecules on the B10.A APC, but not for any possible Ia molecules expressed on the T cell surface, inhibited the GAT response. Overall, these experiments demonstrated that the site of action of the inhibitory effects of anti-Ia antibodies was the Ia molecules expressed on the surface of the antigen-presenting cell.

4. Blocking Experiments Involving Cells from (Responder × Nonresponder)F_1 Animals

The evidence that Ia molecules and *Ir* gene products are functionally related came from anti-Ia blocking experiments with cells from (responder × nonresponder)F_1 animals. These studies were first performed in the guinea pig by Shevach *et al.* (1972) before the discovery of Ia molecules. The antisera used at the time for blocking were anti-histocompatibility sera (strain 2 anti-strain 13 lymphoid cells and vice versa) which were subsequently shown to contain predominantly anti-Ia antibodies (B. Schwartz *et al.*, 1976). The experiment involved the use of two antigens, DNP-GL and GT, and two inbred strains of guinea pigs, 2 and 13, possessing reciprocal response patterns to the two antigens. Thus, strain 2 was a responder to DNP-GL and a nonresponder to GT whereas strain 13 was a responder to

GT and a nonresponder to DNP-GL. The F_1 cross between these two strains responded to both antigens because *Ir* genes are codominant in their expression (see Section V,B,4). When primed T cells from the F_1 were stimulated in culture with DNP-GL, the proliferative response could be inhibited by a 13 anti-2 serum but not a 2 anti-13 serum. In contrast, the response to GT could be inhibited by the 2 anti-13 serum but not the 13 anti-2 serum. The response to PPD, an antigen to which both strains responded, was inhibited partially by each antiserum and almost totally by a mixture of the two antisera. The critical aspect of these results, which have been reproduced by a number of laboratories using different antigens and different species (see R. Schwartz *et al.*, 1978a, for a review), is that only antibodies directed against Ia molecules of the responder strain blocked the response to the antigen, e.g., the GT response was block by a 2 anti-13 serum and the strain 13 is a responder to GT, whereas it was not blocked by a 13 anti-2 serum and the strain 2 is a nonresponder to GT. This observation strongly suggested that the Ia molecule and the *Ir* gene product from the same haplotype were physically associated with each other at the cell surface of the antigen-presenting cell. Although one could imagine a selective expression mechanism that would keep two gene products from the same chromosome physically associated in an F_1, the simplest association, which was postulated at the time of these experiments, was that the two were in fact properties of the same molecule.

The best way to understand this experiment is in terms of the model illustrating the parallel between MHC restriction and *Ir* gene control (Fig. 1). Based on antigen-presentation studies discussed earlier (Section III,C), the F_1 animal has a clone of T cells that is capable of recognizing GT in association with the strain C haplotype-encoded Ia molecules (Ia C), but lacks or cannot stimulate a T cell clone (dotted line) with specificity for GT in association with strain A haplotype-encoded Ia molecules (Ia A). When an anti-strain C Ia antibody is added to this system, the C Ia molecule is masked in some way, preventing the stimulation of the responding T cell specific for GT in association with the C Ia molecule. In contrast, when an anti-strain A Ia antibody is added to this system, there is no effect, because masking the A Ia molecules does not interfere with the stimulation of a T cell participating in the response. The reciprocal outcome for the antigen, DNP-GL, is similarly understood in terms of the response consisting only of T cells specific for DNP-GL in association with strain A Ia molecules. For PPD, which is not under detectable *Ir* gene control, one would predict that both subpopulations of T cells exist in the F_1, each specific for the antigen in association with only one of the two types of Ia molecules (left side of Fig. 1). Thus, anti-Ia antibodies directed against one of the parental forms of the Ia molecule would only partially inhibit the proliferative response. In order to get com-

plete inhibition, antibodies against both parental types of Ia molecules would have to be added. This is exactly what was found (Shevach *et al.*, 1972).

Overall, the demonstration that an *Ir* gene-controlled T cell response could be selectively inhibited by a monoclonal antibody directed against a single determinant on an Ia molecule, which is encoded for by genes linked to the responder allele of the same haplotype, strongly suggested that the Ia molecule and the *Ir* gene product were identical. However, the formal possibility still remained that the Ia molecule and the *Ir* gene product were different structures that were coexpressed in such a manner as to remain physically linked at the surface of the antigen-presenting cell. If such were the case, then anti-Ia antibodies might inhibit the function of an adjacent *Ir* gene product by sterically blocking the molecule or by disturbing its function through some cytoskeletal-induced change.

B. Genetic Complementation of MHC-Linked *Ir* Genes

1. Poly(Glu^{53},Lys^{36},Phe^{11}) (GLPhe)

In the mid 1970s a number of investigators discovered that crossing two low or nonresponder strains could sometimes produce F_1 hybrids that were high responders (for a review see Berzofsky, 1980b). In some cases, such as the myoglobin results discussed in Section II,F (Berzofsky *et al.*, 1979), the high responsiveness resulted simply from the summing of two low responses that were under separate *Ir* gene control and directed at different determinants on the molecule. Because *Ir* genes are codominant (see below) the F_1 was capable of making both responses. In other cases, however, the F_1 was clearly capable of making a response which neither of the parental strains could accomplish (Günther and Rüde, 1975). The best example of this true form of genetic complementation was the response to the synthetic polypeptide poly(Glu^{53},Lys^{36},Phe^{11})$_n$ (GLPhe) (reviewed in Dorf, 1978). Many inbred strains bearing different MHC haplotypes do not make antibodies to GLPhe even when immunized with this antigen in CFA (see Table I). However, crossing two nonresponder strains, such as B10 and B10.A produced F_1 hybrids that yielded large antibody responses to GLPhe (Dorf *et al.*, 1975). This observation suggested the possibility that two distinct *Ir* genes might be required for this response. The proof of this hypothesis came from the identification of responder strains among recombinant mice in which a crossover event had occurred between the chromosomes of the two nonresponder strains (Dorf *et al.*, 1975). The B10.A(3R) (i3 haplotype) and B10.A(5R) (i5 haplotype) recombinants represent such strains. The left half of their MHCs derived from the B10 (*H-2*^{b}) nonresponder and the right half of their MHCs derived from the B10.A(*H-2*^{a}) nonresponder, yet the strains were responders

to GLPhe. These results demonstrated that two genes were involved, one donated by the *H-2*^{b} haplotype, which mapped to the left (centromeric) of the recombinant point and was designated *Ir-GLPhe*-β, and the other donated by the *H-2*^{a} haplotype, which mapped to the right (telomeric) of the recombinant point and was designated *Ir-GLPhe*-α. Examination of other recombinants mapped the genes to the *I-A* subregion for the β gene and the *I-E/C* subregion for the α gene. A similar analysis defined two *Ir* genes controlling the response to GLPhe in [B10.S (*H-2*^{s}) × B10.BR (*H-2*^{k})]F_1 mice, one in *I-A*, the other in *I-E/C*.

Although in many of the examples that have been described the complementation occurs between *I-A* and *I-E/C* subregion encoded genes (so called α–β complementation), in a few cases the complementation turned out to involve genes from the *I-A* subregion of the two haplotypes (so called β–β complementation) (Dorf and Benacerraf, 1975). For example, B10 (*H-2*^{b}) and the GD (*H-2*^{g2}) recombinant were both nonresponders to GLPhe, but the (B10×GD)F_1 was a responder. These two strains only differ in the *I-A* subregion.

The biologic basis of α–β *Ir* gene complementation was worked out by Schwartz and Paul. Initially they demonstrated that the same type of *Ir* gene complementation controlled the T cell proliferative response to GLPhe (R. Schwartz *et al.*, 1976b) (see Table I). This argued that neither of the genes was expressed solely in B lymphocytes since these cells do not usually participate in this assay system. Next, they asked the question whether both genes had to be expressed in the same cell or could one function in the antigen-presenting cell while the other functioned in the T cell? To answer this question, radiation-induced bone marrow chimeras of the type P_1(b.m.) + P_2(b.m.) → F_1(irr) were set up (R. Schwartz *et al.*, 1979b). This chimera creates a situation in which both nonresponder parental cells can interact in a mutually tolerant environment. B10.A(18R)(b.m.) + B10.A(b.m.) → (A×18R)F_1(irr) chimeras failed to respond to GLPhe, although they did respond normally to (T,G)-A- -L and pigeon cytochrome *c*, antigens to which one of the two parental strains were responders. In addition, a chimera composed of a responder and a nonresponder bone marrow donor, the B10.A(3R)(b.m.) + B10.A (b.m.) → (3R×A)F_1(irr), responded to GLPhe, demonstrating that a chimeric environment was permissive for a GLPhe response. These results showed that both responder *Ir-GLPhe* alleles had to be expressed in at least one of the cell types required for a GLPhe response. This suggested that the complementation was intracellular, i.e., at the molecular level.

In order to determine which cell type(s) had to possess both responder alleles, an analysis was begun similar to that described earlier (Sections III,C and E) for localizing the cellular sites of *Ir* gene expression. To investigate

the antigen-presenting cell, primed responder F_1 T cells were stimulated *in vitro* with GLPhe in association with irradiated spleen cells from the F_1 or either of the parental strains (R. Schwartz *et al.*, 1979b). Neither the B10 nor the B10.A nonresponder parental spleen cells, separately or together, could present GLPhe, although they were both competent to present another antigen, PPD. In contrast, the (B10.A × B10)F_1 and the B10.A(5R) recombinant responder strains were both competent to present GLPhe. Thus, the antigen-presenting cell had to possess both responder alleles in order to function. These genes had to be expressed in the same cell, because the mixture of the presenting cells from the two strains, each possessing one of the responder alleles, did not function. These results were completely compatible with the data discussed earlier (Section III,C) on one gene systems and further demonstrated that the antigen-presenting cell was one cellular site of *Ir* gene expression.

In order to determine whether either or both of the responder *Ir-GLPhe* alleles had to be expressed in the T lymphocyte, Longo and Schwartz (1980) returned to the chimera model. The fact that the antigen-presenting cell had to possess both responder alleles could explain why the P_1(b.m.) + P_2(b.m.) → F_1(irr) chimeras failed to respond to GLPhe since the antigen-presenting cells in these animals derived from the nonresponder parental bone marrows. In order to test if this was the only reason for nonresponsiveness, chimeric spleen cells were transferred to lethally irradiated F_1 recipients along with T cell-depleted F_1 bone marrow as a source of responder antigen-presenting cells. The recipients responded to GLPhe, demonstrating that the T cells in the chimera were competent to respond. The proof that neither responder *Ir-GLPhe* allele had to be present in the T cell population came from studies with a B10.A(4R)(b.m.) → [B10.A×B10.A(18R)]F_1(irr) chimera. The B10.A(4R) recombinant (K^kI-A^kI-E^bD^b) is the double negative strain, possessing a nonresponder *Ir-GLPhe* allele at both loci. The T cells from this chimera responded to GLPhe when transferred to an irradiated F_1 along with responder F_1 bone marrow. In addition, the B10.A(4R) T cells responded to (T,G)-A--L and pigeon cytochrome *c*, antigens to which this strain is also a nonresponder. Thus, in agreement with the data discussed earlier (Sections III,E,2 and 3) for helper and DTH-mediating T cells, *Ir* genes, single or dual, did not appear to be expressed in T lymphocytes.

That responsiveness to GLPhe was an acquired characteristic, dependent upon the environment in which the T cells matured, was demonstrated with F_1(b.m.) → P_1(irr). Neither (B10.A×B10)F_1 (b.m.) → B10.A(irr) nor (B10.A×B10)F_1(b.m.) → B10(irr) chimeras responded to GLPhe, although both could respond to other antigens to which the irradiated host was a responder [(T,G)-A--L for B10 and pigeon cytochrome *c* for B10.A]. Thus, the presence of responder antigen-presenting cells, deriving from the F_1

bone marrow, was not sufficient to ensure responsiveness. In addition, the T cells had to differentiate in a responder environment in order to acquire the capacity to respond.

2. *Unique Ia Molecules in F_1 Animals*

Because the parallels between one gene and two gene systems were so great, it seemed reasonable to try and interpret these results in a similar manner. If a unique Ia molecule were to exist in the F_1 that was not present in either parent, then in order for GLPhe to be recognized in association with it, the T cell would have to learn to recognize it as a restriction element, i.e., it would have to be present in the environment in which the T cells matured, and the T cell would have to encounter it during activation, i.e., it would have to be expressed on the surface of the antigen-presenting cell. The first evidence to support the idea of unique F_1 Ia molecules came from the secondary MLR studies of Fathman and Nabholz (1977). In these experiments lymph node T cells from strain A/J mice were primed *in vitro* with irradiated spleen cells from (C57BL/6×A/J)F_1 mice and then rechallenged 2 weeks later with F_1 or B6 parental spleen cells. F_1 spleen cells stimulated a much greater secondary T cell proliferative response than the presumably equally allogeneic B6 spleen cells. A mixture of B6 and A/J parental spleen cells as stimulators was no different than B6 stimulators alone. In contrast, when A/J T cells were first primed with B6 irradiated spleen cells, then both B6 and (B6×A)F_1 spleen cells stimulated a secondary response of equal magnitude. These results suggested that F_1 cells express MLR stimulating determinants that the B6 parental cells lack. These products were shown to be encoded for by genes in the MHC. Subsequently, Fathman *et al.* (1981) were able to isolate clones of T cells specific for these F_1 MLR determinants and to use monoclonal anti-Ia antibodies to inhibit the proliferative response of these clones. The conclusions from these experiments (reviewed in Kimoto *et al.*, 1982) were that unique Ia molecules do exist on the surface of F_1 stimulator cells and that unique T cell clones exist with receptors that are specific for these hybrid Ia molecules.

The biochemical basis for the F_1 specific Ia molecules was first worked out by Jones *et al.* (1978). In studying the structure and expression of Ia molecules by using immunoprecipitation of radiolabeled, detergent extracts from spleen cells, followed by analysis on two-dimensional polyacrylamide gels, they were able to clearly resolve the products of different allelic forms of the Ia molecules encoded by several loci. This enabled them to discover that the cell surface expression of some Ia molecules was under the control of two separate genes mapping in the *I* region of the MHC. One locus, which mapped in the *I-A* subregion, was a structural gene encoding an Ia polypeptide chain. The second locus, which mapped in *I-E*, *I-C*, or *S*, controlled

whether the *I-A*-encoded molecule remained in the cytoplasm or was expressed on the cell surface. Anti-Ia antibodies directed against *I-E*-subregion products precipitated the *I-A*-encoded polypeptide in addition to an *I-E*-encoded polypeptide, suggesting that it was the latter gene product that controlled the cell surface expression of the former. Subsequent studies by Cook *et al.* (1979a), employing tryptic peptide maps, demonstrated that the *I-A*-encoded polypeptide was the β chain of the E_β:E_α Ia molecule and the *I-E*-encoded polypeptide was the α chain. These results suggested that unique Ia molecules could appear on the surface of cells in certain F_1 animals if the hybrids were crosses between a strain such as B6 that produced an E^b_β chain but lacked an E^b_α chain, and a strain such as A/J that expressed an E^a_α chain. Only the (B6×A)F_1 would express E^b_β:E^a_α molecules on the cell surface.

The relevance of these structural studies for *Ir* gene complementation lies in the fact that several of the GLPhe nonresponder strains (B10 and B10.S) do not express an *I-E*-encoded α chain. Therefore, the *I-A*-encoded β chain remains in the cytoplasm, presumably in a nonfunctional state (Jones *et al.*, 1978). However, when these strains are crossed with a strain expressing an E_α chain, e.g., B10.A or B10.BR, the E^b_β or E^s_β chain is brought to the surface. The same is true of the responder recombinant strains, B10.A(3R) and B10.A(5R), which possess *I-E*^{a} and therefore express an E^b_β:E^a_α Ia molecule on the cell surface. Thus, α–β *Ir* gene complementation could be explained if the E^b_β and E^a_α Ia polypeptides were the *Ir* gene products expressed in antigen-presenting cells.

Strong evidence in favor of this hypothesis came from an unexpected correlation between structure and function as further studies were done on Ia molecules and *Ir* gene control. Schwartz *et al.* (1980) were studying the *I-E* subregion products as restriction elements in antigen presentation, using GLPhe as the antigen and B10.A(5R) T cells (*I-A*^{b}:*I-E*^{a}) as the responding population in a proliferation assay. In order to keep the *I-A*^{b} subregion constant and vary the source of the *I-E* subregion, they used as their source of antigen-presenting cells F_1 hybrids between B10 and a variety of other strains with different *I-E* alleles. The presentation pattern they observed did not conform to anything they or others had seen previously. For example, APCs from F_1 strains bearing *I-E*^{d}, *I-E*^{p}, and *I-E*^{r} presented GLPhe to the B10.A(5R) (*I-E*^{a}) T cells, whereas APCs from F_1s bearing *I-E*^{q}, *I-E*^{f}, and *I-E*^{s} did not. Why some allogeneic alleles led to unrestricted interaction while others appeared to result in MHC restriction at the *I-E* subregion was not clear at first. There was no correlation with responder status of the haplotype of the *I-E* donor, e.g., B10.Q (*I*^{q}) was a responder to GLPhe, although there did appear to be a correlation with whether the strain expressed a particular serologic determinant, Ia.7, controlled by a gene in the *I-E* subregion.

The complete clarification of this experiment occurred when further biochemical studies of the E_α molecule were undertaken by several laboratories. Jones *et al.* (1981) found that strains bearing the *H-2* haplotypes *a*, *k*, *d*, *p*, and *r* all expressed an E_α molecule and its associated determinant, Ia.7, whereas strains bearing the *H-2* haplotypes *q*, *f*, and *s* did not express an E_α molecule. Furthermore, Cook *et al.* (1979b) performed tryptic peptide map comparisons on separated E_α and E_β molecules from strains bearing different haplotypes and found that, in contrast to the E_β chains that showed only 50–70% homology, the E_α chains were 85 to 100% homologous. For example, a comparison of E^k_α and E^r_α showed identity between 14 of 15 peptides. More recently some of the genes encoding these molecules have been cloned and sequenced (McNicholas *et al.*, 1982b; Mathis *et al.*, 1983). Again the similarities were striking. For example, the E^a_α and E^d_α alleles differed by 11 nucleotides in their entire coding regions, resulting in only three amino acid differences, two in the second extracellular domain ($\alpha 2$) of the protein and one in the transmembrane region. These observations suggested that the similarity in biological function for antigen presentation of GLPhe was a consequence of the expression of E_α chains that were similar in structure. This conclusion was subsequently supported by the isolation of T cell clones specific for GLPhe and the demonstration that their proliferative response could be inhibited by the monoclonal anti-Ia antibody, Y17 (Ia.m44), which reacts only with $E^{b,k,r,s}_\beta$:$E^{a,k,d,p,r,v}_\alpha$ Ia molecules (Sredni *et al.*, 1981). Thus, GLPhe is usually recognized by T cells in association with the E_β:E_α Ia molecule (B10.Q is the only exception) and the genetic requirements to assemble this molecule would account for the apparent dual *Ir* gene control.

3. *Pigeon Cytochrome c*

Extension of this correlation between structure and function to *Ir* genes was achieved with another antigen, pigeon cytochrome *c*. The response to this antigen was also controlled by two complementing *Ir* genes, one mapping in *I-A*^{k}, the other in *I-E*^{k} (Solinger *et al.*, 1979). In contrast to GLPhe, very few strains responded to pigeon cytochrome *c*. Therefore, an examination of complementation for responsiveness was possible using F_1 crosses between the nonresponder B10.A(4R) strain, which only expresses the E^k_β molecule (*I-A*^{k}), and nonresponder strains expressing E_α chains encoded for by different alleles. Strains expressing $E^{a,d,p,k,\text{ or } r}_\alpha$ would all complement for pigeon cytochrome *c* responsiveness, whereas strains not expressing an E_α chain, such as B10 or B10.S, did not complement (Matis *et al.*, 1982). Similar results were obtained for antigen presentation when primed B10.A (E^k_β:E^a_α) responder T cells were presented pigeon cytochrome *c* on F_1 spleen cells derived from crosses between B10.A(4R), providing E^k_β, and strains express-

ing or not expressing an E_α chain. Thus, the structurally similar E_α chains appeared to be biologically equivalent in both antigen-presentation function and associated *Ir* gene control, suggesting that the E_β:E_α Ia molecule was the *Ir* gene product.

However, there was one strain, the B10.PL (*H-2*^{u}), that expressed an Ia.7-positive E_α chain yet failed to complement with the *I-A*^{k} allele to produce responsiveness to pigeon cytochrome *c* (Matis *et al.*, 1982). In addition, when crossed with B10.A(4R), it failed to produce an F_1 spleen cell population capable of presenting the antigen to B10.A T cells. At first these results appeared to contradict the Ia polypeptide chain complementation model to explain *Ir* gene complementation, because the Ia.7-bearing E^u_α chain could combine with the E^u_β chain to result in an immune response to GLPhe, while this same E^u_α chain did not appear to complement with the E^k_β chain, i.e., [B10.A(4R)×B10.PL]F_1 was a nonresponder to pigeon cytochrome *c*. The reason for this discrepancy was subsequently shown to be the result of quantitative differences in Ia molecule expression. A careful set of biochemical and quantitative adsorption studies by McNicholas *et al.* (1982a) revealed that the E^k_β:E^u_α Ia molecule was present on the surface of [B10.A(4R)×B10.PL]F_1 spleen cells, but in one-eighth the amount normally detected on homozygous B10.BR cells (E^k_β:E^k_α). The normal amount detected by quantitative immunofluorescence was $3–5\times10^4$ molecules per cell. The amount found on [B10.A(4R)×B10.PL]F_1 spleen cells was only one-fourth of the total amount of E^k_β:E_α Ia molecules detected on the surface of other F_1 cells. A two-dimensional polyacrylamide gel electrophoresis analysis suggested that the reason for this low expression of the E^k_β:E^u_α Ia molecule was a preferential association of E^u_β and E^u_α molecules with each other in the cytoplasm of the F_1 cells. The structural basis for this preferential chain association is still unknown.

This quantitative difference in Ia molecule expression was then shown by Matis *et al.* (1982) to have a functional correlate in antigen-presentation studies. When a pigeon cytochrome *c*-specific B10.A long-term T cell line was presented with antigen in the presence of different irradiated spleen cell populations, [B10.A(4R)×B10.PL]F_1 cells (E^k_β:E^u_α) required a 10-fold higher dose of antigen to give the same stimulation as an equal number of [B10.A(4R)×B10.D2]F_1 cells (E^k_β:E^d_α). Furthermore, at any given antigen concentration, the monoclonal anti-Ia antibody, Ia.m44, which reacts with the E^k_β:$E^{d,u}_\alpha$ but *not* the E^u_β:E^u_α Ia molecule had a greater inhibitory effect on the pigeon cytochrome *c* response in the presence of [B10.A(4R)×B10.PL]F_1 spleen cells than in the presence of [B10.A(4R)×B10.D2]F_1 spleen cells. These results demonstrated that the E^k_β:E^u_α Ia molecule could function as a restriction element for presentation of pigeon cytochrome *c* to B10.A T cells, making the correlation between E_α expression and antigen presentation

perfect. In addition, they demonstrated that the quantitative level of expression of an Ia molecule at the cell surface could affect antigen-presentation function. The fact that high doses of antigen can compensate for a low number of Ia molecules and that this low amount is more sensitive to inhibition with an anti-Ia antibody suggested that the T cell response was dependent upon the concentration of both the antigen (Ag) and the Ia molecule (Ia) on the surface of the antigen-presenting cell, i.e., $d\text{CPM}/dt = k[\text{Ag}][\text{Ia}]$, where k is the proportionality constant and CPM is the proliferative response. Recent quantitative studies of T cell activation (Matis *et al.*, 1983a), in which the Ia molecule and the antigen concentrations were systematically varied, clearly demonstrated this product relationship. How the quantitative deficiency in E^k_β:E^u_α Ia molecule expression results in the nonresponsiveness of the [B10.A(4R)×B10.PL]F_1 mouse to pigeon cytochrome *c* will be discussed in Section VI,D.

4. *Gene Dosage Effects*

Another interesting quantitative observation to come out of the studies on *Ir* gene complementation was the clear demonstration of gene dosage effects. Early studies on the response to GLPhe showed that (B10.BR×B10.S)(H-2^k×H-2^s)F_1 hybrids produced significantly lower antibody and T cell proliferative responses than the responding recombinant strains B10.S(9R) and B10.HTT (Dorf *et al.*, 1976; R. Schwartz *et al.*, 1976b). This observation was initially interpreted by Dorf and Benacerraf as a cis–trans effect, i.e., that the positioning of the responder α and β alleles on the same chromosome was more effective than positioning these alleles on opposite chromosomes. The alternative interpretation was a gene dosage effect, i.e., that the concentration of molecules needed for a response was significantly reduced in the F_1 animal because the genes were codominantly expressed. The latter was thought to be eliminated by the full response of an (H-2^s×H-2^f)F_1, the (B10.HTT×A.CA)F_1 strain. This is because the H-2^f haplotype (I-A^f, I-E^-) was thought not to contain a GLPhe responder allele at either the *Ir-GLPhe* α or β locus, since (H-2^f×H-2^a)F_1 mice (which tests for the contribution of a β locus) and (H-2^f×H-2^b)F_1 mice (which tests for the contribution of an α locus) were nonresponders to GLPhe. However, subsequent studies (Dorf *et al.*, 1979) demonstrated that β–β complementation occurred between the H-2^s and H-2^f haplotypes (I-A^s complemented with I-A^f to produce unique F_1 Ia molecules, see below), which could have masked a gene dosage effect. Thus, the full response seen in the (B10.HTT×A.CA)F_1 could have been the sum of the (H-2^s×H-2^f) β–β complementation and a reduced level of α–β complementation.

The strong possibility that this was the case was raised by the studies of R. Schwartz *et al.* (1979a) on the synthetic polypeptide poly(Glu^{57},Lys^{38},Tyr^5)$_n$

(GLT^5). In attempts to show that the T cell proliferative response to GLT^5 was under the control of two complementing MHC-linked *Ir* genes, these authors discovered conditions of preparation of the polymer in which the B10.A(5R) recombinant was a high responder to the antigen but the (B10.A×B10)F_1 mouse was a low responder. They proved that the poor responsiveness of the latter strain was due to a gene dosage effect by showing that the [B10.A(5R)×B10.A(4R)]F_1 was also a low responder to GLT^5. This hybrid represents a cross between a strain bearing both high responder alleles, B10.A(5R) (*Ir-GLT* β^b and *Ir-GLT* α^k), and a strain bearing both low responder alleles, B10.A(4R) (*Ir-GLT*-β^k and *Ir-GLT* α^b). In the case of this F_1, both high responder alleles were on the same chromosome (cis), whereas in the case of the (B10.A×B10)F_1, the two high responder alleles were on opposite chromosomes (trans). Thus, whether the two genes were in cis or trans, the maximal proliferative response was about 15–20% of that seen with cells from the B10.A(5R) mouse. This result demonstrated that the chromosomal localization of the genes was unimportant and that what mattered was the number of copies of the genes.

Subsequent experiments by Dorf *et al.* (1979) confirmed this conclusion for GLPhe. They mated the B10.S(8R) strain, which possesses both low responder *Ir-GLPhe*-β^k and *Ir-GLPhe*-α^s alleles, with the high responder B10.S(9R) (*Ir-GLPhe*-β^s and *Ir-GLPhe*-α^k), and immunized the F_1 with GLPhe. This F_1 was an intermediate responder, similar to the (B10.A×B10.S) and (B10.BR×B10.S)F_1s. Thus, in the absence of β–β complementation, a gene dosage effect was clearly seen.

The presence of gene dosage effects in both the *Ir-GLT* and *Ir-GLPhe* systems strongly suggested that both the high responder and low responder alleles were codominantly expressed. This conclusion was supported by an earlier statistical analysis of Ebringer *et al.* (1976) who analyzed all the quantitative data on the genetic control of the immune response available in the literature up to 1975. They used a genetic technique for normalizing the data, called a dominance index (*d*), in which

$$d = \frac{(F_1 - L)}{1/2(H - L)} - 1$$

where H, L, and F_1 are the quantitative antibody responses in the high responder (H), low responder (L), and (H×L)F_1 animals, respectively. For complete dominance (F_1 = H), $d = +1$, and for complete recessiveness (F_1 = L), $d = -1$. If $d = 0$ the system is codominant. The mean value for all 91 experiments involving 1527 animals was $+0.0076 \pm 0.153$, which was not significantly different from zero. The distribution seemed to be symmetrical about the mean, even if one rejected data whose absolute value of *d* was

greater than 2. The authors concluded from their analysis that *Ir* gene systems were codominant. However, this conclusion was challenged because the analysis lumped together experiments involving responses whose genetic basis was unknown with those involving known MHC-linked *Ir* genes, and included experiments involving complementing gene systems where the F_1 was the only responder ($d > 1$). An analysis of only those data derived from established unigenic *Ir* gene-controlled systems gave a d between 0 and +1 depending on which experiments were analyzed. Thus, the question of dominance vs codominance of *Ir* genes in any individual case was not clearly answered until the complementation experiments were completed.

If *Ir* gene products are the Ia molecules, then codominant expression has to be the correct interpretation, since both parental histocompatibility molecules can always be detected in an F_1. However, the quantitative aspects of the cell surface expression of these molecules might easily be responsible for obscuring the true nature of the expression of the genes at these loci. The reasons for this should already be clear, given the extensive discussion of complementation experiments presented in this section. In some cases gene dosage effects are masked because of the selective associations between certain allelic forms of particular α and β chains. For example, the [B10.A(4R)×B10.PL]F_1 gave as good a response to GLPhe as the B10.PL parent since the quantity of the E^u_β:E^u_α Ia molecule was only slightly reduced in this F_1. In other cases, gene dosage effects are masked by complementation events occurring in the F_1. For example, the antibody response to (T,G)-A--L in (H-2^a×H-2^b)F_1 mice was only slightly lower than that of the high responder strain. This can now be explained by β–β complementation, which occurs in the F_1. Solinger and Schwartz (1980) demonstrated that the core polymer, G-A--L, was immunogenic in the F_1 even though neither parental strain could respond to it. In addition, Kimoto and Fathman (1980) have isolated (T,G)-A--L specific T cell clones from (H-2^a×H-2^b)F_1 animals and found that some of them are specific for (T,G)-A--L only in association with F_1 antigen-presenting cells. Their analysis of the basis for this F_1 specificity (Kimoto *et al.*, 1982) has revealed that the α and β chains encoded in the I-A^b and I-A^a subregions associate in all 4 combinations, A^a_α:A^a_β, A^b_α:A^b_β, A^a_α:A^b_β, A^b_α:A^a_β to give rise to the 2 unique Ia molecules found only in the F_1. Thus, β–β *Ir* gene complementation could be explained by the existence of these F_1 specific Ia molecules and the requirement of T cells to recognize certain antigenic determinants, such as G-A--L, only in association with them. In turn, the response of such unique F_1 T cells might compensate for the gene dosage effect. Thus, it seems likely that gene dosage effects normally occur all the time, but that they are obscured by these other phenomena.

The biochemical basis of a gene dosage effect is the reduction in the

amount of protein made from a structural gene when the number of copies of that gene is reduced. In an F_1 animal, this is normally a reduction of 50% for a single genetic locus. However, in the case of Ia molecules, which are encoded by two genetic loci, one might expect the reduction to be 75%, since the concentration of each reactant, α and β, would be cut in half. This type of analysis, however, assumes that no regulation of gene transcription occurs. A more likely scenario is that the total amount of Ia molecules in the cell is maintained at a certain level. In this case, the amount of any one form of the Ia molecule and the concomitant biological effect would depend on the nature and preferential associations of the individual chains. For example, assume that $E^k_\beta{:}E^k_\alpha$ is the Ia molecule required to respond to a particular antigen and that $E^d_\beta{:}E^d_\alpha$ and the hybrid Ia molecules, $E^k_\beta{:}E^d_\alpha$ and $E^d_\beta{:}E^k_\alpha$, are not functional. In a $(k\times d)F_1$ animal, if the pairwise association between the two α and β chains is random, then the concentration of the necessary $E^k_\beta{:}E^k_\alpha$ Ia molecule would be reduced to ¼ of that found in a homozygous strain. Given the quantitative effects mentioned earlier for the cytochrome *c* system, a 75% reduction in surface concentration might account for a loss in biological activity. In contrast, if the α chains of *k* and *d* were functionally equivalent, as is the case with most E_α molecules, then two of the four combinations, $E^k_\beta{:}E^k_\alpha$ and $E^k_\beta{:}E^d_\alpha$, would be functional and the concentration of the required Ia molecule would only be reduced by ½. This 50% difference might or might not be detected functionally. A comparison of the B10.A and $(B10.A\times B10.D2)F_1$ spleen cells for their ability to present pigeon cytochrome *c* showed only a slight difference (Matis *et al.*, 1982). On the other hand, when certain chains show marked degrees of preference for each other and only one combination leads to responsiveness, then differences are either easily detectable (e.g., $E^k_\beta{:}E^u_\alpha$ for pigeon cytochrome *c*) or impossible to detect (e.g., $E^u_\beta{:}E^u_\alpha$ for GLPhe) depending on which Ia molecule is required for responsiveness.

Ongoing molecular biology experiments should eventually allow one to predict the outcome of a response in an F_1 animal once one knows in addition which Ia molecules can be used by the antigen as a restriction element. R. N. Germain (personal communication) has begun transfecting into L cells Ia genes in different allelic combinations and measuring the degree of cell surface expression. This eventually will provide a hierarchy of the different preferential chain associations. For example, $A^b_\beta{:}A^k_\alpha$ was found to be expressed almost as readily as either of the homozygous pairs, $A^b_\beta{:}A^b_\alpha$ and $A^k_\beta{:}A^k_\alpha$. In contrast, $A^b_\beta{:}A^d_\alpha$ showed significantly lower expression and $A^d_\beta{:}A^k_\alpha$ and $A^k_\beta{:}A^d_\alpha$ were hardly expressed at all. These preliminary results correlate quite nicely with known functional results involving β–β complementation, i.e., several examples are known involving the $A^b_\beta{:}A^k_\alpha$ molecule, only one is known involving the $A^b_\alpha{:}A^d_\alpha$ molecule, and no examples have been

reported in $(k \times d)F_1$ hybrids. In addition, Germain has discovered that certain A/E combinations, such as A^d_β:E^k_α, can assemble and be expressed on the cell surface. This surprising result suggests that previously unexpected forms of complementation might end up explaining certain puzzling *Ir* gene effects (see discussion of the *I-B* subregion in Section VI,C,4,e).

In sum, it appears as if all the varied aspects of *Ir* gene complementation can be clearly understood if the Ia molecules are in fact the *Ir* gene products. However, it should be noted that all of the data presented merely establish a correlation, both qualitative and quantitative, between Ia molecule expression and *Ir* gene function. This does not constitute proof of their identity.

C. The *xid* Gene and Ia.W39

Another example of a correlation between the qualitative expression of an Ia molecule and *Ir* gene control was demonstrated using the CBA/N mouse, which carries the *xid* gene on its X chromosome (for a review of the biology and genetics of the CBA/N mouse, see Scher, 1981). In addition to the absence of a B cell subpopulation, which is manifest in this strain and F_1 males derived from crosses involving CBA/N females, Huber (1979) discovered that $(CBA/N \times B6)F_1$ females express an Ia determinant, Ia.W39, that is not found on spleen cells from $(CBA/N \times B6)F_1$ males. Ia.W39 was shown to be a private determinant of the $H\text{-}2^b$ haplotype, requiring for expression a gene(s) in the $I\text{-}A^b$ subregion. It was selectively expressed on the Lyb5 positive subset of B cells, and was absent on cells from newborn females in addition to adult males carrying the *xid* gene.

Rosenwasser and Huber (1981) subsequently discovered that the $(CBA/N \times B6)F_1$ males could not respond when immunized with beef insulin, whereas the $(CBA/N \times B6)F_1$ females could respond. The reciprocal $(B6 \times CBA/N)F_1$ male, which does not carry the *xid* gene, also responded to beef insulin. In addition to the *Ir* gene defect, the $(CBA/N \times B6)F_1$ males manifested an antigen-presentation defect. Thus, T cells from $(CBA/N \times B6)F_1$ females immunized with beef insulin could be stimulated to proliferate to beef insulin on antigen-presenting cells from F_1 females but not on antigen-presenting cells from F_1 males bearing the *xid* gene. This was a selective defect of these male cells since they were capable of presenting TNP-OVA. Similarly a comparison of neonatal and adult $(CBA/N \times B6)F_1$ female antigen-presenting cells demonstrated that 8-day-old macrophages were incapable of presenting beef insulin, although they presented TNP-OVA as well as did adult macrophages.

Biochemical studies on Ia.W39 by Huber *et al.* (1981) demonstrated that both the $(CBA/N \times B6)F_1$ males and females synthesize the Ia molecule bearing this determinant. However, only in adult F_1 females is the molecule

found on the cell surface. In adult F_1 males and neonatal females, the Ia molecule is found in the cytoplasm, but similar to the E_β polypeptide chain involved in α–β complementation, in this location the molecule is not functional. The relationship between the Ia.W39-bearing Ia molecule and the A_β:A_α Ia molecule is not clear. All that is known is that antisera (anti-Ia.W39) recognizing the former molecule will precipitate some but not all of the A_β:A_α molecules recognized by an anti-*I*-A^b antisera, while experiments with tunicamycin have demonstrated that the Ia.W39-bearing molecule is not a posttranslational N-linked carbohydrate modification of an A_β:A_α Ia molecule. However, whatever its physical nature, the data clearly suggest that this Ia molecule must be expressed on the surface of an antigen-presenting cell in order for these cells to be able to present beef insulin to T cells. Animals that cannot do this are nonresponders to beef insulin. Thus, again we see the correlation between Ia molecule expression, antigen presentation, and *Ir* gene function.

At this point it would seem that the correlative evidence is overwhelmingly in favor of an identity between Ia molecules and *Ir* gene products. However, the epistatic effect of the CBA/N *xid* gene serves as a good example as to why this type of correlation does not constitute proof. The *xid* gene product appears to regulate the expression of certain Ia molecules on the surface of B cells and macrophages, but clearly the Ia molecules, whose structural genes map in the MHC, are not the *xid* gene product(s) that is encoded on the X chromosome. The same relationship might be true for *Ir* genes. Although they are closely linked to the structural genes coding for Ia molecules, they could be nearby regulatory genes affecting the expression of Ia molecules. The only way to prove an identity between Ia molecules and *Ir* gene products is to identify a mouse strain bearing a mutation in the structural gene coding for an Ia molecule and then to demonstrate that this mutation also affects *Ir* gene function.

D. *I* Region Mutations

1. The B6.C-H-2^{bm12}(bm12) Mutation

The B6.C-*H*-2^{bm12} (bm12) mutant was first detected by Melvold and Kohn (1976) as a spontaneous mutation in a (C57BL/6×BALB/c)F_1 mouse using a screening assay involving reciprocal tail skin grafting. The mutation was shown to be in the *H*-2^b haplotype by acceptance or rejection of parental skin grafts and therefore made homozygous by backcrossing to the C57BL/6Kh parent. Both the inbred bm12 mutant strain and the congenic C57BL/6 parent strain ("wild type") reject each other's skin grafts in 14–16 days. This indicates that the mutation has occurred in the major histocompatibility complex and is both the gain and loss type, i.e., the mutant expresses antigens that are recognized by the C57BL/6 immune system as allogeneic (gain), and

the mutant immune system recognizes antigens that are expressed by the C57BL/6 as allogeneic, antigens that the mutant has lost and therefore is not tolerant to (loss).

Complementation studies done by McKenzie *et al.* (1979, 1980) in which the bm12 was crossed with different strains to determine which would produce F_1 hybrids that did not reject C57BL/6 skin grafts, demonstrated that the mutation had occurred in the *I-A*^{b} subregion. Thus, if bm12 was crossed with C57BL/6, the F_1 would not reject C57BL/6 skin grafts (control). However, if the bm12 was crossed with B10.A(4R) or D2.GD, which possess *b* alleles in all of the MHC regions accept for the *K* region and the *I-A* subregion, then the F_1 did reject C57BL/6 skin grafts. The reciprocal recombinant, B10.A(5R), with *b* alleles in *I-A* and *K*, when crossed with C57BL/6 did not reject C57BL/6 skin grafts. These complementation results mapped the mutation to the *K* region or the *I-A* subregion. The elimination of the *K* region was done with known *H-2K* mutants, bm1, bm5, bm6, and bm8. All of these produced F_1 hybrids that would not reject C57BL/6 skin grafts, suggesting that the bm12 mutation was not in the same locus as the other mutants. The formal demonstration that K^b would not complement was performed by Hansen *et al.* (1980) using the B10.MBR strain ($K^bI^kS^kD^q$). (bm12×B10.MBR)F_1 mice rejected C57BL/10 skin grafts. This result, in conjunction with the [bm12×B10.A(4R)]F_1 result, located the mutation to the *I-A*^{b} subregion.

This conclusion was confirmed in serological studies with anti-Ia alloantisera by McKenzie *et al.* (1979, 1980). Reactions of bm12 spleen cells with anti-Ia antibodies specific for *I-A*^{b}-encoded determinants, Ia.3, Ia.8, Ia.9, Ia.15, and Ia.20, were greatly reduced or completely absent compared to the reaction of these reagents with normal C57BL/6 cells. The results were the same by either microcytotoxicity or adsorption assays. In contrast, using alloantisera specific for determinants on K^b-encoded molecule(s), no differences were detected between the mutant and the wild type. These observations were confirmed by Lafuse *et al.* (1981), using LPS-induced B cell blasts as the targets, which allowed a clearer distinction of the effects on individual Ia determinants. In this study the expression of Ia.3, 9, 15, and 20 was only slightly reduced on bm12 cells, whereas Ia.8 was completely gone. This result suggested that the bm12 strain might carry a structural mutation with selective alteration of a particular part of the Ia molecule.

Definitive proof of the nature of the mutation in the bm12 was obtained from an analysis of tryptic peptide maps of the Ia molecules. *I-A*^{b}-encoded Ia molecules were isolated by McKean *et al.* (1981) from spleen cells of the bm12 and C57BL/6 strains using a monoclonal anti-Ia.15 immunoadsorbent. The internally labeled ([^{3}H]Arg, [^{3}H]Lys) α and β chains were separated, digested with trypsin, and the peptide fragments compared using C_{18} re-

verse-phase high-pressure liquid chromatography. The tryptic peptide maps for the A_α chains from bm12 and C57BL/6 were identical. However, A_β chain maps showed a number of significant differences. Each strain displayed 3 unique peptides, out of approximately 23, that were lacking in the map from the other strain. Thus, the site of the mutation appeared to be in the gene encoding the β polypeptide chain of the A_β:A_α Ia molecule.

The exact nature of the mutation was determined using molecular biology techniques. McIntyre and Seidman (1984) cloned and sequenced the gene encoding the bm12 A_β chain. Three nucleotide differences were found, each resulting in an amino acid substitution: Phe for Ile at position 67, Gln for Arg at position 70, and Lys for Thr at position 71. Thus, the bm12 was not a point mutation, but rather represented a more complex genetic event. Recent data from two laboratories (Widera and Flavell, 1984; Mengle-Gaw *et al.*, 1984) have suggested that what has occurred is genetic exchange of information from other class II genes. Sequence analysis of the E^b_β gene showed an identical nucleotide sequence to the bm12 A_β gene in the region encoding amino acids 62 to 75. Thus, the E^b_β gene might have been the donor of a short piece of DNA in a recombinational event such as gene conversion or double reciprocal recombination. Despite the complexity of the event, these data establish that the mutation has occurred in a structural gene that codes for an Ia molecule.

Knowing this we can now ask what effects this mutation has had on *Ir* gene function. A number of laboratories have immunized the bm12 with a variety of antigens and looked for gain or loss of responsiveness by measuring antibody, plaque-forming cell (PFC), and/or T lymphocyte proliferative responses. The critical findings are as follows. In contrast to the C57BL/6 congenic strain, the bm12 mutant has lost the ability to respond to beef insulin (Lin *et al.*, 1981) and the α chain of human hemoglobin (Krco *et al.*, 1981) as measured by T cell proliferation, while the mutant has gained the ability to respond to sheep insulin (Hochman and Huber, 1984) as measured by T cell proliferation and to the synthetic polypeptide, poly-$(Glu^{50},Tyr^{50})_n$(GT), as measured by a PFC response both *in vivo* and *in vitro* (Lei *et al.*, 1982). Responsiveness to other antigens, such as (T,G)-A--L, (Phe,G)-A--L, (H,G)-A--L, calf skin collagen, and ovalbumin was unaffected. In one interesting case, Lei *et al.* (1982) demonstrated that the effects on responsiveness were split along functional lines. The T cell proliferative response of the bm12 mutant to poly$(Glu^{60},Ala^{30},Tyr^{10})_n$(GAT) was comparable to that of the C57BL/6 mouse but the PFC response was completely gone and the delayed-type hypersensitivity response was significantly reduced. Finally, the bm12 mutant has also lost the ability to generate a cytotoxic T cell response against the male specific antigen, H-Y (Michaelides *et al.*, 1981). Although this is a more complex immune response involving

both helper and cytotoxic T lymphocytes, the fact that Michaelides *et al.* (1981) were able to show that cells from male bm12s could serve as targets for cytotoxic T cells obtained from C57BL/6 females suggested that the bm12 defect was not in what the cytotoxic T cell could recognize (i.e., H-Y in association with the class I molecule serving as the restriction element), but rather in the generation of helper T cells. Overall, these experiments demonstrated quite convincingly that the Ir phenotype of the bm12 is different from that of the C57BL/6. Thus, the A_β chain of the *I-A* encoded Ia molecule, which is the only known difference between these two strains, appears to be the *Ir* gene product.

One subtle but important caveat on this conclusion is the question of whether the mutant strain only possesses an alteration in the gene coding for A_β. Spontaneous mutations can occur at all loci in the genome given enough time. This phenomenon of genetic drift raises the possibility that mutant genes at loci other than the one detected are contributing to the overall change in phenotype. This problem is greatly reduced in the bm12 because the mutation arose in a (B6×BALB/c)F_1 and was transferred to a B6 background by backcrossing to the C56BL/6Kh parental line to make the bm12 coisogenic with B6. However, at each step the mutant phenotype was selected for. Therefore, if more than one genetic locus was responsible, they all might have been preserved. The only way one can prove that this has not occurred is to isolate the gene, in both its native and mutant forms, and get them expressed in another (hopefully neutral) host. Although this is a difficult task, it might not be impossible. The recent development of the technology for creating transgenic mice (Brinster *et al.*, 1981) should allow one to transfer both the responder native gene and the nonresponder mutant gene into an MHC congenic nonresponder host. If no unexpected form of complementation occurs, the Ir phenotype of these transgenic mice would provide the definitive proof that the MHC-encoded molecules are the sole determiners of this type of immune response control.

Interestingly, a number of the nonresponder characteristics of the bm12 mutant disappeared when the strain was mated to B10.A. The (B10.A×bm12)F_1 was a responder to beef insulin (Lin *et al.*, 1981) and the H-Y antigen (Michaelides *et al.*, 1981) even though B10.A is also a nonresponder to both antigens. This complementation can be explained in an analogous way to previous β–β complementations (see Section V,B,4) by postulating that the A^k_β molecule of the B10.A combines with an A^b_α Ia molecule of the bm12 to create a hybrid A^k_β:A^b_α Ia molecule that is capable of functioning as an *Ir* gene product that allows or facilitates a response to beef insulin and the H-Y antigen. Consistent with this interpretation is the fact that the Ia.W39 determinant, which was associated with immune responsiveness to beef insulin in (CBA/N× B6)F_1 mice, is absent from both the

bm12 and the (B10.A×bm12)F_1, even though one strain is a nonresponder to the antigen and the other is a responder (Huber *et al.*, 1982). This suggests that Ia.W39 is a determinant requiring the A^b_β chain for expression and that the (B10.A × bm12)F_1 uses a different, unique F_1 determinant to respond to beef insulin. The latter has been shown to be the case by Reske-Kunz and Rüde (1982). In addition, they showed that the antigenic determinant on beef insulin that was being recognized was different from the one recognized by the B6 mouse (Reske-Kunz and Rüde, 1984).

The bm12 mutation has also established that Ia molecules are the structural gene products required for a number of other *I* region encoded phenomena. For example, McKenzie *et al.* (1979) have shown that C57BL/6 T cells manifest a strong mixed leukocyte response (MLR) when stimulated with bm12 spleen cells and vice versa. This result demonstrated that the A^b_β and A^{bm12}_β polypeptide chains are molecules expressing lymphocyte activating determinants (LAD) that provide the antigenic stimulus from an MLR. Perhaps the most important additional *I* region-mediated phenomenon to be attributed to Ia molecules, as a consequence of experiments with the bm12 mutant, is the phenomenon of MHC restriction. Fathman *et al.* (1981) used T cell clones from (B6×A)F_1 mice immunized with (T,G)-A--L to study the genetic requirements for antigen presentation in the initiation of a T cell proliferative response. Some clones required (B6×A)F_1 antigen-presenting cells in order to respond to (T,G)-A--L. Genetic mapping studies using [B10.A(4R)×B6] and [B10.MBR×A.AL]F_1s demonstrated that *I* region products from both the *I*-A^b and *I*-A^k subregions were required for stimulation of the clones. When the bm12 mutant was substituted for B6 in generating the F_1, the (bm12×B10.A)F_1 presenting cells would stimulate a (T,G)-A--L-specific proliferative response from some T cell clones but not others. These results were interpreted to mean that the F_1-specific T cell clones recognize (T,G)-A--L in association with the unique A^k_β:A^b_α and A^b_β:A^k_α F_1 combinatorial Ia molecules and that the mutant, which has an altered A^{bm12}_β chain, formed an altered A^{bm12}_β:A^k_α Ia molecule. This was confirmed by Beck *et al.* (1983) using monoclonal anti-A^k_β, A^k_α, and A^b_β antibodies, which selectively blocked the proliferative responses of these clones. Thus, any T cell clone restricted to recognizing (T,G)-A--L in association with the A^b_β:A^k_α Ia molecule would not recognize the A^{bm12}_β:A^k_α Ia molecule. In contrast, any T cell clone restricted to recognizing (T,G)-A--L in association with the A^k_β:A^b_α Ia molecule did not have its restriction element altered by the mutation and, therefore, could be stimulated with (T,G)-A--L in the presence of (bm12×B10.A)F_1 presenting cells. Since the bm12 and B6 congenic strains only differ in their A_β chains, the conclusion from this work was that the A_β chain is at least part of the restriction element involved in antigen presentation to T lymphocytes.

The combined use of the bm12 mutant to cement the relationship between *Ir* gene products and Ia molecules as restriction elements is nicely demonstrated in the experiments of Lin *et al.* (1981). They crossed the C57BL/6 responder strain to beef insulin with the bm12 nonresponder mutant to produce the responder strain, (B6×bm12)F_1. When primed T cells from the F_1 were presented beef insulin in association with various antigen-presenting cells, spleen cells from the responder F_1 and B6 parent stimulated a proliferative response, but spleen cells from the nonresponder bm12 parent did not, although the latter presented (T,G)-A--L, OVA, and PPD quite well. Thus, nonresponder presenting cells could not stimulate T cell clones specific for beef insulin in association with responder Ia molecules. The failure in this case was clearly due to a structural alteration in its Ia molecule caused by the mutation in its A_β gene. This same mutation resulted in an *Ir* gene defect, which could now be clearly seen to be a failure to stimulate T cell clones specific for beef insulin in association with A_β^{bm12}:A_α^b Ia molecules. The mechanisms by which this might occur will be discussed in the next section. Now, however, it is only important to conclude that the Ia molecule is both the restriction element for certain subpopulations of T cells and the *Ir* gene product that influences the immune response to certain antigens in association with that restriction element. Thus, MHC restriction and *Ir* gene control (as well as alloreactivity) are pleiomorphic manifestations of the same gene product. The common link among these phenomena is that they all represent different aspects of T cell recognition. When viewed in this light it can be seen that *Ir* gene effects are simply a manifestation of the determinant specific nature of MHC-restricted recognition (Fig. 1).

2. *Mutations Generated in Vitro*

The bm12 mutation is the only spontaneous mutation in the *I* region to be identified thus far. Given how powerful it has been in improving our understanding of the biology of the immune system, it is not surprising that other mutants are desired to confirm and expand our knowledge. The usual approach for finding such mutants is by skin grafting, a procedure that appears not to readily identify *I* region mutations. As a consequence, several investigators have begun to explore other approaches. One of the most promising has been the selection *in vitro* of antigen-presenting cells bearing altered Ia molecules. Glimcher *et al.* (1983a) developed an antigen-presenting cell possessing only one H-2^k-bearing chromosome, TA3, by fusing normal B cells from an (H-2^k×H-2^d)-bearing F_1 strain to an H-2^d-bearing B cell tumor, M12, that they had previously shown (Glimcher *et al.*, 1982) was capable of presenting antigens to T cells. The resulting somatic hybrid could present pigeon cytochrome *c*, GAT and hen eggwhite lysozyme (HEL) to T cell clones capable of recognizing these antigens in association with H-2^k-en-

coded Ia molecules. The original parental $H\text{-}2^d$-bearing tumor could not present these antigens. Using this somatic cell hybrid, they next selected for variants bearing altered Ia^k molecules by mutagenizing with ethyl methane sulfonate, killing the cell population with one anti-Ia monoclonal antibody and complement, and then positively selecting the remaining cells for expression of the same Ia molecule by fluorescence staining with a second anti-Ia monoclonal and enriching for such cells by electronic cell sorting on a fluorescence-activated cell sorter. After two rounds of this negative and positive selection, they were able to clone out a significant number of variants that possessed alterations in only one of the two Ia molecules of the $H\text{-}2^k$ haplotype, as assessed by staining with different anti-Ia monoclonals. These cells were then tested for antigen presentation function in an IL-2 release assay using T cell hybridomas specific for various antigens in association with $I\text{-}A^k$- and $I\text{-}E^k$-encoded Ia molecules. Mutants, such as A8, were shown to have lost the ability to present HEL and to have a reduced capacity to present GAT, both of which require the A^k_β:A^k_α Ia molecule for T cell recognition (Glimcher *et al.*, 1983a). In contrast, presentation of pigeon cytochrome *c*, which requires the E_β:E_α Ia molecule, remained completely intact. The partial presentation of GAT supported the serologic data in demonstrating that the A_β:A_α Ia molecule was still expressed on the cell surface, but in an altered form. Thus, the mutation was similar in character to the bm12 mutant, and the experiments supported the conclusion that Ia molecules are restriction elements.

The first of these mutant cell lines to have its Ia genes cloned and sequenced was A19 (Brown *et al.*, 1985). This variant had lost the ability to stimulate T cell hybridomas specific for PPD and KLH in association with A^k_β:A^k_α, while retaining the ability to stimulate T cell hybridomas specific for GAT in association with A^k_β:A^k_α, HEL in association with E^k_β:E^k_α, and several autoreactive hybridomas specific for A^k_β:A^k_α (Glimcher *et al.*, 1983b). In addition, it split A^k_β:A^k_α-OVA- and HEL-specific hybridomas into two groups; some were stimulated by A19, others were not. The nucleotide sequence of the A_β gene revealed a single nucleotide difference (G→A), which changed the glutamic acid at position 67 to a lysine. Interestingly, this amino acid substitution occurred in the same area of the Ia molecule affected by the bm12 mutation. These results suggested that this region, referred to by Mengle-Gaw and McDevitt (1983) as the third hypervariable region, plays an important role in MHC-restricted, antigen recognition. Proof that this point mutation, and not other alterations in the genome, was responsible for the biological effects was achieved by transfecting the gene into L cells, selecting for high expressors on the cell sorter, and showing that the L cells displayed the same pattern of antigen presentation as the original A19 mutant line (Brown *et al.*, 1985). Thus, a selective alteration in the β chain of an Ia molecule had clearly altered the biological activity of this molecule in T cell activation.

Although this approach is still in its infancy, it is already obvious how powerful it will be for probing the structure and function of Ia molecules. Eventually, the molecular biology techniques of site-directed mutagenesis and gene transfection should allow the full characterization of every portion of the Ia molecule without imposing any selection procedures. In addition, it should be possible to introduce these altered genes into the pronuclei of fertilized eggs for expression in a way that would allow one to examine their Ir effects (transgenic mice). Thus, this area of immunology will certainly be an exciting one to watch unfold in the next few years.

VI. Theories of *Ir* Gene Function

A. Introduction

The demonstration that Ia molecules are the *Ir* gene products was an important conceptual breakthrough in our understanding of *Ir* genes. It allowed one to integrate *Ir* gene function into the general framework of T cell recognition and thus to merge two originally disparate areas in cellular immunology, MHC restriction and *Ir* genes. However, this unification did not answer the question of how *Ir* gene products function, i.e., what is it about a particular Ia molecule that results in a specific pattern of nonresponsiveness to a given set of antigens (Table I)? To answer this question, one must turn to the biological system and ask what fails to occur when an animal is a nonresponder to a particular antigen. What the experiments outlined in Sections II–V have revealed is that T lymphocytes with a particular specificity fail to be detected in most immunological assays. These lymphocytes should have receptors with the property of recognizing a particular antigen in association with the Ia molecule encoded by the nonresponder MHC haplotype. The problem does not lie in the recognition of either of these components alone, since (responder × nonresponder)F_1 hybrids can respond to the same antigen in association with Ia molecules encoded by the responder MHC haplotype or to other antigens in association with the nonresponder Ia molecule. Only the unique combination of that particular antigen and that particular Ia molecule is not recognized. Thus, the question of how *Ir* gene products function leads us to consider the nature of the T cell repertoire and the requirements for T cell activation.

T cell activation is a "three body" problem involving a T cell receptor, an Ia molecule, and an antigen. These three components must interact in some temporal sequence and with some spatial relationship in order to transmit a signal to the T cell and have it respond. In considering ways in which the structure of the Ia molecule might influence the activation process, it is clear

that two possible avenues are open to it. One is through physical interaction with antigen; the other is through physical interaction with the T cell receptor. Those models that attempt to explain *Ir* gene function solely in terms of Ia molecule–antigen interactions have been termed presentational models. They essentially postulate that the Ia molecule has some specificity for antigen and that only antigens that can be positively recognized by the Ia molecules will associate with (binding model) or be processed by (processing model) them. Thus, *Ir* gene defects result from the failure of a particular Ia molecule to interact with a particular antigen. In such models no constraints are placed on the T cell repertoire, which, in fact, could be totipotent.

In contrast, if one chooses to place the emphasis on Ia molecule–T cell receptor interactions to explain *Ir* gene function, then constraints on the T cell repertoire are unavoidable. If T cells could recognize all combinations of antigenic determinants and Ia molecules, either through germline-encoded receptors and/or through somatic mutation to achieve all specificities, then T cells capable of recognizing antigen in association with nonresponder Ia molecules should exist and respond. The experimental failure to see such T cells then can only be attributed in such repertoire constraint models to the absence of T cell clones with that particular dual specificity. This has been postulated to occur in a number of ways. The simplest and most straightforward model is to postulate that the genes that encode the T cell receptor are limited in number. In this case, the defect would be the failure to assemble a receptor with specificity for the particular combination of antigen and nonresponder Ia molecule. Alternatively, one could postulate that the T cell repertoire is initially totipotent, but that somatic events secondarily delete certain T cell clones, for example, during the generation of tolerance to self molecules. In this case, the T cells that are specific for nonresponder Ia molecules and antigen would be missing because their receptors also interact with self Ia molecules, an event leading to clonal deletion.

Another avenue of repertoire modulation, which is open to those scientists who believe in the ontological acquisition of MHC restriction, is thymic selection (see R. Schwartz, 1984, for a review of the two theories on repertoire acquisition). This event is thought to occur in the absence of antigen, and, therefore, can only explain *Ir* gene effects in terms of T cell receptor, Ia molecule interactions. In this case, the repertoire is limited by the positive selection of T cell clones with specificity for self Ia molecules. During this process a large number of T cell clones from a totipotent repertoire would be lost, including those clones that would have been capable of recognizing the antigen in association with the nonresponder Ia molecule, due to a failure to positively select them. Alternatively, in models that start with a limited germline-encoded repertoire and generate receptor diversity by somatic mutation, such as the original model proposed by Jerne (1971), the clones

with specificity for the nonresponder Ia molecule and antigen might not be generated, because of the limited diversity achieved by the somatic mutation process in a finite amount of time.

Finally, it is possible that a large responsive T cell repertoire might be limited by the introduction of antigen-specific suppressor T cells. These cells have been found in nonresponder animals in a number of cases, although the mechanism by which Ia molecule polymorphism might determine the presence or absence of these cells has seldom been clearly espoused. Nonetheless, it is possible that suppressor cells could selectively inhibit those T cells that recognize the antigen in association with nonresponder Ia molecules.

The goal in this section of the review will be to outline for the reader in detail the models that have been proposed to explain *Ir* gene function. In addition, any experimental evidence, pro or con, bearing on these models will be presented. However, the reader should be warned in advance that there have not been any definitive experiments that would allow one to exclusively choose one model over any of the others. In fact, it is likely that these models are not mutually exclusive and that in some situations one model will provide the correct interpretation of the results, while in a different situation another model will prove to be the correct interpretation.

B. Antigen Presentation Models

1. Antigen Processing

a. Models. Levine and Benacerraf (1964) were the first to consider the possibility that *Ir* genes controlled the ability of macrophages to "process" antigen. In this type of model, the *Ir* gene products were viewed as degradative enzymes that could breakdown some antigens but not others. This idea derived from the early observations on synthetic polypeptides composed of D-amino acids (reviewed in Sela, 1966). These molecules were poorly immunogenic as well as poorly degraded. Therefore, it was assumed that degradation was an essential step that was required to convert an antigen into an immunogenic form (Gottlieb and Schwartz, 1972). However, experimental attempts to demonstrate a difference between macrophages from responder and nonresponder guinea pigs in their ability to take up and degrade various hapten–poly-L-lysine conjugates failed to detect any differences (Levine and Benacerraf, 1964). Thus, the early workers in the field abandoned this idea.

More recently several investigators have returned to the idea of *Ir* gene function as an antigen-processing event. In the current models, as proposed by Blanden *et al.* (1976) and by Rothenberg (1978), the *Ir* gene products have been postulated to be glycosyltransferases (or other similar types of enzymes) that modify the antigen in such a way as to make it recognizable by T cells. This is not as unreasonable an assumption as it might seem at first glance since

the gene encoding a known glycosyltransferase, neuraminidase 1, has been mapped to the murine MHC, between *Slp* and *H-2D* (Figueroa *et al.*, 1982). In one variation of this type of model, a dual receptor is envisioned on the T cell, with one unit specific for the antigen, the other specific for the oligosaccharide created by the glycosyltransferases. In the other variation, a single receptor or set of receptors recognizes the oligosaccharide modification(s) made to the antigen. In both cases the oligosaccharide-recognizing moiety on the T cell is postulated to be the glycosyltransferase or a closely related family of molecules that have lost enzymatic activity. For the most part, this type of model has fallen by the wayside as structural studies on MHC-encoded molecules have progressed. The main reason for this is because no one has reported finding glycosyltransferase activity in any of the purified preparations of these class I and II molecules.

b. Evidence for Antigen Processing. The early evidence in favor of any kind of antigen processing by macrophages was mostly indirect. For example, primary and secondary T cell responses to soluble protein antigens could be elicited by enzymatically or chemically cleaved fragments of the molecules (reviewed in Katz, 1977b). One experiment of this type, which was performed in my laboratory (Solinger *et al.*, 1979), involved immunization of B10.A mice with pigeon cytochrome *c*. When the primed T cells were challenged *in vitro* with either the native molecule or the C-terminal cyanogen bromide cleavage fragment of cytochrome *c*, the latter actually stimulated a T cell proliferative response at lower antigen concentrations, when compared on a molar basis, than the intact immunogen. The dose–response curve for the fragment was shifted to a lower dose by approximately 4-fold. Work with synthetic fragments showed that molecules as small as the C-terminal decapeptide, residues 94–103, of a cross-reacting insect cytochrome *c*, were still capable of stimulating a proliferative response (Hansburg *et al.*, 1983a). In addition, most of the small peptides were immunogenic, eliciting a T cell population that was similar in specificity to the population elicited with the intact cytochrome *c* protein. These results clearly showed that artifically processed molecules could function as complete antigens; however, they did not prove that intact protein molecules must be processed in order to make them immunogenic.

Evidence that processing must occur, at least for some particulate antigens, was first obtained from kinetic experiments comparing uptake, ingestion, catabolism, and antigen presentation by macrophages (for a review of this area, see Unanue, 1984). Using ^{125}I-labeled *Listeria monocytogenes*, Ziegler and Unanue (1981) were able to show that attachment of the organisms (uptake) to the outer surface of macrophage membranes was complete in 5–10 minutes after exposure. Ingestion by phagocytosis followed rapidly, reaching completion in 40 minutes. In contrast, antigen presentation, de-

fined as the ability of primed T lymphocytes to recognize and bind to macrophage monolayers, did not begin until 20 minutes after *Listeria* were added, and did not reach a maximum for 60 minutes. The appearance of presentation seemed to correlate with catabolism of the antigen, as defined by the release of trichloroacetic acid-soluble ^{125}I label, which began with ingestion at 5–10 minutes and was 35% complete at 1 hour. Even more convincing were experiments in which catabolism and presentation were completely dissociated from uptake on the basis of temperature and energy dependence. Antigen presentation could be reduced by 85% if the assay was carried out at 4°C. At this temperature catabolism was not detected at all, although uptake was unaffected. Similarly, in the presence of sodium azide, which inhibits oxidative metabolism, and 2-deoxyglucose, which inhibits glycolytic metabolism, antigen presentation was reduced by 80%, catabolism was reduced by 50%, but uptake was unaffected. Furthermore, experiments with ammonium chloride and chloroquine (Ziegler and Unanue, 1982), compounds that inhibit lysosomal function, showed that catabolism and presentation could be substantially inhibited without affecting either uptake or ingestion. These results suggested that *Listeria* organisms must be catabolized by macrophages before any of their antigens could be recognized by T cells.

A major breakthrough in this area occurred when Ziegler and Unanue (1981) introduced paraformaldehyde fixation of macrophages as a way to inhibit antigen processing. Treatment of macrophages with a 1% solution of paraformaldehyde after uptake of *Listeria*, but before the onset of catabolism, prevented the binding of immune T lymphocytes. In contrast, treatment 1 hour after the macrophages were exposed to the *Listeria* organisms only reduced the binding by about 40%. Thus, aldehyde fixation did not prevent T cell binding, only the earlier processing steps. Shimonkevitz *et al.* (1983) followed up on these studies by applying them to a simpler system, the response of T cell hybridomas specific for ovalbumin (OVA) in association with A^d_β:A^d_α on the surface of an antigen-presenting B cell tumor, A20. Light fixation of A20 with glutaraldehyde prevented it from presenting intact OVA, although prepulsing the cells with OVA (i.e., exposure of the cells to the antigen, followed by washing away of the unbound OVA) and then fixing them did not block antigen presentation. These authors reasoned that if antigen processing involved only catabolism, then one should be able to bypass this requirement by enzymatically or chemically degrading the antigen. This turned out to be true. Trypsin and cyanogen bromide fragments of OVA were presented by glutaraldehyde-fixed A20 cells, while intact OVA was not. These results suggested that degradation of the antigen was necessary and sufficient for antigen processing of soluble protein antigens.

This seminal observation was confirmed by Allen and Unanue (1984) for

lysozyme, Streicher *et al.* (1984) for myoglobin, and Kovac and Schwartz (1985) for cytochrome *c*. The only modification in interpretation centered around the need for antigen catabolism. Whereas Shimonkevitz *et al.* (1983) had tested denatured OVA and found it insufficiently altered to function with aldehyde-fixed presenting cells, Allen and Unanue (1984) and Streicher *et al.* (1984) found cases in which simply altering the three-dimensional structure of the molecule by reduction and alkylation or S-methylation was sufficient to allow the antigen to be presented by fixed or chloroquine-pretreated antigen-presenting cells, respectively. These observations suggested that the critical step in antigen processing was not degradation per se, but rather an unfolding of the molecule to expose the antigenic determinant for recognition (although the possibility of improved uptake onto the membrane of the presenting cell was not excluded). This was most clearly demonstrated in the case of pigeon cytochrome *c* for which the T cell antigenic determinant had been well defined (Kovac and Schwartz, 1985). The lysine at position 99, which was identified as the most likely residue to be contacting the T cell receptor, was found to be involved in the intact molecule in a salt bridge with the glutamic acid at position 61. Cleavage of the molecule with cyanogen bromide to give the fragment 66–104 allowed this fragment to be presented by aldehyde-fixed cells. In contrast, cleavage of the molecule with hydrogen bromide gave fragment 60–104, which could not be presented by aldehyde-fixed cells. This was interpreted to mean that removal of the Glu-61 allowed the C-terminal portion of the molecule to rearrange in such a way that the lysine 99 could then interact with the T cell receptor.

Although a significant amount of evidence has accumulated to support the idea of processing by antigen-presenting cells, no evidence has been published so far that supports the notion that Ia molecules are responsible for such processing. In fact, Ziegler and Unanue (1981) have shown that anti-Ia antibodies, which are capable of inhibiting T cell binding to macrophages, did not inhibit uptake and catabolism of *Listeria*. This result suggests that the Ia molecules are not involved in the processing of this particular antigen. One would think that since it is known that Ia molecules are the *Ir* gene products, it would be a simple task to determine whether they have any enzymatic activity. These molecules can be easily isolated, have been completely sequenced, and can be inserted into liposomes (B. Schwartz and Cullen, 1978). Yet, to date there have been no reports that Ia molecules have any enzymatic activity whatsoever, let alone differences in activity for different allelic products. In addition, computer searches of the gene and protein data banks have not revealed any sequence homologies between Ia molecules and known proteins with enzymatic activity (Norcross and Kanehisa, 1985). Thus, without any experimental data to support it, the idea that *Ir* gene products exert their antigen-specific effects by some processing

mechanism is viewed by most workers in the field as an unlikely explanation for *Ir* gene function.

c. Constraints Imposed on the Immune Response by Antigen Processing. Nonetheless, the mere existence of a processing step can impose limitations on the immune response. Depending on the nature of the catabolic enzymes which exist and the tertiary structure of the molecule being degraded, only certain fragments will be produced. If these fragments are not able to be recognized by the immune system because of an *Ir* gene defect, then no response will occur. Conversely, other determinants, which would be capable of being recognized by T cells in association with an Ia molecule, might be destroyed during the degradation, thus preventing them from eliciting an immune response. The best experimental example of this kind of phenomenon comes from the work of Thomas *et al.* (1985) on the response to fibrinopeptide in guinea pigs. Strain 2 guinea pigs were found to be responders to the ββ 1–14 peptide from fibrinogen, whereas strain 13 guinea pigs were nonresponders. However, if the C-terminal arginine was removed to give ββ 1–13, then strain 13 guinea pigs were found to be responders to this synthetic peptide whereas strain 2 guinea pigs were nonresponders. The $(2\times13)F_1$ was a responder to both peptides because of the codominant expression of *Ir* genes. The interesting experimental result was obtained when $(2\times13)F_1$ guinea pigs were immunized with both peptides. A T cell proliferative response could be obtained to both peptides *in vitro* if the correct peptide was added to the cultures. However, if native fibrinogen was added to the cultures, then only the T cells specific for ββ 1–14 in association with strain 2 Ia molecules were stimulated. Thus, even though the potential for an immune response to ββ 1–13 existed, the inability of the presenting cells to degrade the fibrinogen in a way that created ββ 1–13 prevented this response from occurring.

A similar interpretation has been applied to experimental results obtained with beef cytochrome *c* in the mouse (Buchmüller and Corradin, 1982). DBA/2 mice (I^d) when immunized with apocytochrome *c* gave rise to T cell clones that could be stimulated by apocytochrome *c* but not by native cytochrome, even though the latter contains all the amino acids possessed by the former. In contrast, T cells from C57BL/6 mice (I^b) were stimulated equally well by native and apocytochrome *c*. Therefore, antigen processing can influence the immune response in what appears to be an MHC haplotype-specific manner, even though the Ia molecules are not themselves involved in the processing steps. This is because processing is temporally an earlier event than recognition and thus it can limit the nature and quantity of fragments available for the immune system to see, even though there is no difference in the processing of the antigen in macrophages from high and low responder animals.

2. *Antigen Binding*

a. Determinant Selection. The antigen-binding type of model was first proposed by Rosenthal (1978) in what was termed the determinant selection model, and subsequently championed by Benacerraf (1978) in a slightly altered form. The essential feature of the model is that antigen and Ia molecules physically interact, i.e., they have a certain binding affinity for each other in the absence of the T cell receptor. Different Ia molecules have different affinities for the same antigen, and in the original extreme form of the model, nonresponder Ia molecules do not bind to the antigen at all. As a consequence of the binding of antigen by an Ia molecule, a limitation is placed on what other determinants on the antigen are available for the T cell receptor to recognize. In other words, determinant selection by the antigen-presenting cell precludes certain T cell clones from being stimulated, even though they might be present. Thus, the model places no constraints on the T cell repertoire or the T cell receptor.

The original experimental basis for this model was the series of classical experiments of Shevach and Rosenthal (1973) with (responder × nonresponder)F_1 guinea pigs. Primed T cells from these animals would not respond to antigen when it was presented on nonresponder macrophages. This was interpreted to mean a defect in presentation. However, as we have detailed earlier (Section IV), the subsequent discovery of MHC restriction and the existence of two separate T cell populations in the F_1, each capable of recognizing the antigen only in association with one parental Ia molecule, meant that the failure of nonresponder macrophages to present antigen could be interpreted instead as an absence of the T cell subpopulation specific for the antigen in association with the nonresponder Ia molecule. The inability to distinguish between these two interpretations of the data prevents the experiments from being used to support a presentation model for *Ir* gene function.

Clearly, the best evidence one could obtain to support an antigen-presentation model would be to isolate Ia molecules and demonstrate that those from responder animals bind the antigen, whereas those from nonresponder animals do not. This experiment has not been reported. The next best experiment would be to demonstrate a selective association between certain antigens and particular Ia molecules. At the functional level such associations are already quite clear. For example, in the mouse, GLPhe and pigeon cytochrome *c* are only recognized in association with the E_β:E_α Ia molecule, whereas (T,G)-A--L and GAT are only recognized in association with the A_β:A_α Ia molecule (see Nagy *et al.*, 1981, for a summary of this topic). This conclusion is supported by antigen-presentation experiments with animals bearing MHC recombinant chromosomes, as well as by blocking experi-

ments with monoclonal anti-Ia antibodies. In fact, as discussed earlier (Section V,A,2), it has been demonstrated that monoclonal antibodies directed at a single Ia molecule will inhibit some immune responses involving this Ia molecule but not others. This result suggests the possibility of site-specific interactions between antigen and determinants on the Ia molecule. However, all of these experiments are only indirect evidence for a physical association between antigen and the Ia molecule. They could all equally well be explained on the basis of limitations in the T cell repertoire.

b. Ia Molecule–Antigen Interaction in the Pigeon Cytochrome c Response. Recently, however, experiments with T cell clones have uncovered a new phenomenon, selective cross-reactivities or degeneracies in the T cell repertoire for the recognition of MHC-encoded molecules (Heber-Katz *et al.*, 1982; Hedrick *et al.*, 1982; Matis *et al.*, 1983b). These clones, with their limited violation of the rule of MHC restriction, have allowed antigen-presentation experiments to be performed in the presence of two different Ia molecules. Since the T cell receptor could be held constant (in contrast to the earlier experiments of Shevach and Rosenthal), an unambiguous assessment could be made of the Ia molecule's role in determining antigen-specificity. Heber-Katz *et al.* (1982, 1983) found that T cell hybridomas specific for moth cytochrome *c* fragment 81–103, obtained from either the B10.A or B10.A(5R) strains, had very similar recognition capacity. For example, clones from both strains could respond to moth cytochrome *c* fragment 81–103 in association with either B10.A or B10.A(5R) antigen-presenting cells [B10.A expresses E^k_β:E^k_α whereas B10.A(5R) expresses E^b_β:E^k_α as the restricting element for cytochrome *c* recognition]. However, the fine specificity of antigen recognition was not identical. For example, the clones would respond to pigeon cytochrome *c* fragment 81–104 in association with B10.A antigen-presenting cells, but not B10.A(5R) antigen-presenting cells. Since the T cell receptor in these experiments was fixed, because of the clonal nature of the responding population, the change in specificity observed with a change in Ia molecule (E^k_β for E^b_β) must be attributed to an antigen-Ia molecule interaction.[2]

The interesting aspect of this particular system is that the B10.A strain is a

[2] The assumption was made in these experiments that a T cell clone has only a single receptor. If, however, a clone expresses both an antigen-specific receptor and an independent chain that accounts for alloreactivity, a model proposed by Williamson (1980), then the cytochrome *c* data could be explained without requiring an antigen–Ia molecule interaction. The recent structural studies done on the antigen-specific receptor (see Section VI,C,1) as well as blocking studies by Kaye and Janeway (1984) with monoclonal antireceptor antibodies, suggest that the antigen and the allo-Ia receptors are overlapping. However, definitive proof that only a single receptor with both specificities exists will require transfection experiments with the α and β chain T cell receptor genes.

responder to pigeon cytochrome *c* whereas the B10.A(5R) strain is a nonresponder. Thus, it seemed possible from the clonal observations that one aspect of the *Ir* gene control of the response to pigeon cytochrome *c* was at the level of antigen presentation. To prove this, studies on T cell populations were performed (Heber-Katz *et al.*, 1983). B10.A(5R) mice were immunized with moth cytochrome *c* fragment 81–103 and primed T cells taken from the peritoneal cavity, a population known to be low in alloreactive cells. These T cells responded to moth cytochrome *c* fragment 81–103, but not to pigeon cytochrome *c* fragment 81–104, when these antigens were presented on syngeneic B10.A(5R) antigen-presenting cells. However, taking advantage of the knowledge gained from the clones, when allogeneic B10.A antigen-presenting cells were used, a strong proliferative response was observed (above the small mixed lymphocyte response) to both pigeon and moth cytochrome *c* fragments. Thus, the B10.A(5R) nonresponder strain did contain T cells capable of recognizing pigeon cytochrome *c*, but only in the context of responder B10.A Ia molecules. Therefore, one reason the B10.A(5R) is a nonresponder is because its E_β:E_α Ia molecules cannot interact favorably with pigeon cytochrome *c* to present it to these particular moth cytochrome *c*-activated B10.A(5R) T cells. Note, however, that the failure to activate these clones cannot be the only reason that B10.A(5R) is a nonresponder strain. It also must fail to activate T cell clones specific for pigeon cytochrome *c* in association with E_β:E_α (or, for that matter, also A_β:A_α) Ia molecules. Whether such clones do not exist in the B10.A(5R) mouse, or whether they do exist but cannot be stimulated because of the failure of the antigen and the Ia molecules to interact, cannot be determined from these experiments.

The fact that Ia molecules and antigen appear to interact during T cell activation is consistent with an antigen-presentation model of *Ir* gene function, but does not prove it. These two molecules might only come together in the presence of the T cell receptor, and, if so, might not physically interact. In this case, the influence of one molecule on the binding of the other could occur through an allosteric change (or an induced fit) in the receptor (Fig. 2A). To explore the differences between this more complex T cell receptor allosteric (or induced fit) model and an antigen-presentation model requires an analysis at the molecular level. One of the essential characteristics of any antigen-presentation model is that there must exist *two* separate sites on the antigen involved in stimulating the T cell. One of these sites interacts with the receptor on the T lymphocyte, the other interacts with the Ia molecule on the presenting cell. In contrast, a T cell receptor allosteric (or induced fit) model only requires a single determinant on the antigen, one that interacts with the T lymphocyte receptor (see Cleveland and Erlanger, 1984, for a discussion of this type of model).

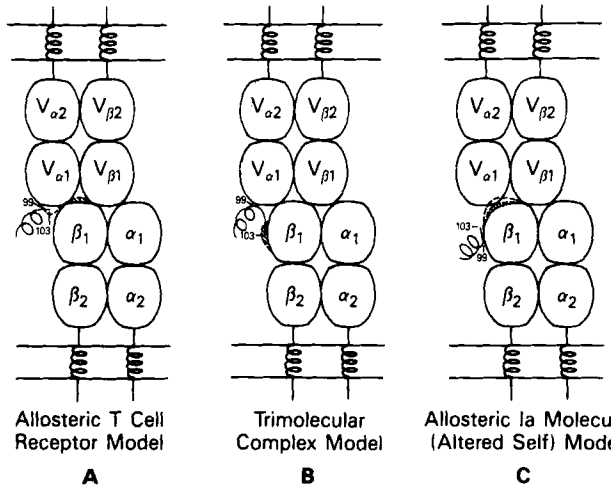


FIG. 2. Molecular models of antigen-specific T cell recognition. The biological data summarized in the text (Section VI,B,2,b) argue that the recognition of antigen and the Ia molecule by the T cell receptor does not occur in an independent fashion. The allelic form of the Ia molecule clearly influences the specificity of the receptor for antigen. These results can be explained by any of the three categories of models depicted in this figure. (A) A representative diagram of a T cell receptor allosteric (or induced fit) model in which the allelic form of the Ia molecule (depicted as the dotted bulge on the β_1 domain with hatched lines) stabilizes or alters the shape of the antigen combining site (depicted as the clear bulge on the $V_{\alpha 1}$ domain). In the extreme case, antigen would not be recognized at all. In pigeon cytochrome *c* recognition, as shown here, the antigen contacts the $V_{\alpha 1}$ domain of the T cell receptor and not the β_1 domain of the Ia molecule. However, only the binding of the C-terminal residues of the cytochrome *c* peptide (designated 103), and not the binding of residue 99, is influenced by the allosteric effect or the induced change. The trimolecular complex model, shown in (B), postulates that all three components in the system physically interact during T cell activation. This model requires that the antigen have two different subsites, one that contacts the T cell receptor (99) and one that contacts the Ia molecule (the C-terminal residues of cytochrome *c*). An allelic change in the Ia molecule could either disturb the interaction with the T cell receptor, leading to MHC restriction (*not* shown in this diagram), or disturb the interaction with the antigen. This latter possibility is shown here for the cytochrome *c* system as a hatched bulge in the β_1 domain interfering with the binding of the C-terminal residues to the Ia molecule. This type of model can be subdivided into two forms. The one in which the antigen and the Ia molecule interact with low affinity and are brought into contact only in the presence of the T cell receptor is usually referred to as the trimolecular complex model. The alternative, in which the antigen and the Ia molecule interact with high affinity, and thus can bind together in the absence of the receptor, is usually referred to as the determinant selection model. During activation of the T cell, as shown here, these two models are indistinguishable. Finally, in (C) is shown a traditional altered self model in which the antigen only interacts with the Ia molecule and not the T cell receptor. The receptor recognizes unique allosteric states or induced changes in the Ia molecule that are stabilized or induced by the antigen. In the extreme example shown here, the β_1 domain of the Ia molecule does not contact the $V_{\alpha 1}$ domain at all in the absence of antigen. Binding of antigen to the β_1 domain distorts this domain or stabilizes it in an alternative state

Studies on the structural requirements for antigenicity and immunogenicity of pigeon cytochrome *c* fragment 81–104 have provided experimental evidence for the existence of two functionally distinct sites on this molecule (Hansburg *et al.*, 1981, 1983a,b). When B10.A mice were immunized with pigeon fragment 81–104 and their T cells stimulated *in vitro* with homologous cytochrome *c* fragments 81–103 and 81–104 from several other species, a pattern of cross-reactions was observed which allowed one to identify certain critical residues that were necessary to initiate a proliferative response. In particular, several insect cytochrome *c* fragments (from *Manduca* and *Hematobia*) stimulated a heteroclitic response, i.e., they were more potent than the immunogen, whereas the fragment from the closely related Pekin duck and chicken cytochromes *c* stimulated poorly. This pattern, which was termed an antigenic hierarchy, served as a fingerprint to identify the specificity of the T cell response. A surprising observation was made, however, when the pigeon fragment 81–104 was chemically derivatized with methylacetimidate. This procedure converted the 5 lysines in the pigeon fragment 81–104 to their *N*-ϵ-acetimidyl derivative (Am pigeon fragment 81–104). The immune response to the derivatized cytochrome *c* appeared to involve a different set of T cells, because immunization with Am pigeon fragment 81–104 produced a population of B10.A T cells that did not respond very well when stimulated with the native pigeon fragment 81–104. Similarly, B10.A T cells primed to native pigeon fragment 81–104 were poorly cross-stimulated by the Am pigeon fragment 81–104 (about 1000-fold less potent). However, an analysis of the critical residues required for stimulating Am pigeon fragment 81–104 primed T cells by testing for cross-stimulation with methylacetimidyl derivatives of cytochrome *c* fragments from other species, revealed a pattern that was very similar to that observed for T cells primed with native pigeon 81–104. Again, the *Manduca* and *Hematobia* amidinated insect cytochromes *c* stimulated heteroclitic responses whereas the closely related Am duck cytochrome *c* fragment 81–104 was a poor antigen. Thus, two poorly cross-reactive immune responses appeared to share recognition of a similar antigenic site on both Am and native pigeon fragments. However, the fact that they were poorly cross-reactive

(hatched bulge) so that it contacts the $V_{\alpha 1}$ domain. Changing the allelic form of the Ia molecule usually leads to MHC restriction (not shown), but in the special case of E^k_β:E^a_α and E^b_β:E^a_α in the cytochrome *c* system, only a slight difference in shape exists (again not shown). However, the magnitude of this shape difference is amplified by the allosteric (or induced fit) effect created in the E^b_β:E^a_α Ia molecule by the C-terminal residues of pigeon cytochrome *c* (clear bulge depicted by dotted lines overlapping the $V_{\alpha 1}$ domain). Residue 99 in this case makes an equal contribution to the altered configurations created in both Ia molecules (hatched bulge). For a more detailed discussion of these three types of models see Rosenthal (1978), Benacerraf (1978), Sherman (1982), Heber-Katz *et al.* (1983), Cleveland and Erlanger (1984), and Schwartz (1985).

suggested that a second site on the antigen, designated the Lys/Am Lys site, could be recognized in a discriminating fashion. This discrimination could only be a property of T cell recognition because it was a learned event involving memory of Lys versus Am Lys. In contrast, the site(s) responsible for the antigenic hierarchy was constant and, therefore, need not be recognized by a T cell receptor. Instead, it could be involved in an interaction with the Ia molecule.

Evidence that the site responsible for the antigenic hierarchy produced its effect by interacting with the Ia molecule came from a study of the *Ir* genes involved in the T cell proliferative responses. B10.A mice were responders to both native and Am pigeon fragment 81–104, while B10.A(5R) mice were low or nonresponders. In contrast, B10.A(5R) mice were responders to both native and Am moth cytochrome *c* fragments 81–103. In addition, antigen-presentation experiments using Am pigeon and Am moth cytochrome *c* fragments gave results identical to the native fragments described earlier, i.e., B10.A antigen-presenting cells presented either Am pigeon or Am moth fragments to B10.A T cells primed to Am pigeon fragment 81–104 or B10.A(5R) T cells primed to Am moth fragment 81–103, but B10.A(5R) antigen-presenting cells presented Am moth fragment 81–103 to these two T cell populations much better than they presented Am-pigeon fragment 81–104. These results demonstrated that all aspects of Ia molecule involvement in the response to pigeon and moth cytochrome *c* fragments in the two strains were unaffected by amidination. Thus, Ia molecule interaction at one antigen site could be functionally separated from T cell receptor interaction at another (Am Lys/Lys) site. In contrast, the antigenic hierarchy was affected by changes in the Ia molecule; B10.A mice responded to pigeon and moth cytochrome *c* fragments (both Am and native), but B10.A(5R) mice only responded to the moth fragment 81–103. This correlation suggested that the residues that interact with the Ia molecule might also be the ones that determined the antigenic strength in the hierarchy. Note that these results are formally analogous to the classic hapten-PLL experiments in guinea pigs carried out by Levine *et al.* (1963a) discussed earlier (see Section II,B). In this case the hapten served as the site on the molecule for which the T cells were specific and the PLL served as the site that interacted with the Ia molecule.

The exact locations of the cytochrome *c* residues involved in each of the two putative sites were identified using synthetic peptide analogs of the pigeon and moth fragments, containing single amino acid substitutions. The Am Lys/Lys site was identified as residue 99 because a Gln substituted for a Lys at this position produced a peptide that elicited a new, non-cross-reacting T cell population in B10.A mice without changing antigenic strength (i.e., the moth analog was more potent than the pigeon analog), *Ir*

gene control [i.e., the pigeon analog was immunogenic in B10.A but not B10.A(5R) mice], or antigen presentation of the pigeon analog by B10.A and not B10.A(5R) spleen cells. Furthermore, amidination had no effect on the ability of the fragment to stimulate, because the Gln was unaffected by this derivatization procedure. In contrast, the residue responsible for differences in antigen presentation by B10.A and B10.A(5R) spleen cells as well as differences in antigenic strength and *Ir* gene control was identified as the presence or absence of an alanine at position 103. A synthetic analog of pigeon fragment 81–104, in which the alanine 103 was deleted, moving the C-terminal lysine next to threonine 102 (as is found in the moth fragment), behaved exactly like moth fragment 81–103. It was heteroclitic in stimulation for B10.A T cells primed to pigeon fragment 81–104 and it was immunogenic in B10.A(5R) mice. Thus, the two functionally distinguishable sites appeared to be only 4 residues apart. In fact, their β carbon atoms might be only 5–6 Å apart, as studies by Pincus *et al.* (1983), using conformational energy calculations and energy minimization techniques, suggested that the most favored conformation for the moth peptide in a nonpolar environment was an alpha helix.

c. Molecular Models of T Cell Activation. It is difficult to imagine how two proteins as large as an Ia molecule and a T cell receptor could independently interact with two parts of a small antigenic peptide separated by only 5–6 Å. Thus, some investigators (Cleveland and Erlanger, 1984) prefer to try and interpret the "two-subsite" data in terms of a T cell receptor allosteric (or induced fit) model in which the two (or more) residues are part of a single antigenic determinant interacting with one combining site on the T cell receptor (see Fig. 2A). In this case, however, one must hypothesize that binding of the Ia molecule to a separate location on the receptor only influences allosterically one part of the antigen-combining site, and that the T cell repertoire cannot adapt to the allosteric effect of the B10.A(5R) Ia molecule by substituting a different receptor that would allow this strain to respond to pigeon cytochrome *c* (even though it can adapt to changes at residue 99). An alternative model, involving physical interaction of the Ia molecule and the antigen is easiest to visualize with a T cell receptor in which the two chains are linked to form a single combining site (Fig. 2B), although other versions are possible (see Norcross and Kanehisa, 1985). In this case, however, it would seem that most of the binding energy for the formation of the trimolecular complex would have to come from the Ia molecule–T cell receptor interaction, because the sizes of these two molecules would lead to multiple points of contact. If so, it is difficult to imagine how the Am Lys/Lys site could exert so much influence on the specificity of the response, since it is only a single contact residue (99). Finally, another model compatible with the cytochrome *c* data that also involves a physical interaction between the

Ia molecule and the antigen is shown in Fig. 2C. In this model the antigen does not interact with the T cell receptor at all! Rather it binds to the Ia molecule and alters its conformation by an allosteric (or induced fit) mechanism. In this case, the Am Lys/Lys site at position 99 would be responsible for the allosteric effect. The T cell receptor then recognizes the induced or stabilized alteration in the Ia molecule that occurs subsequent to the interaction of the Ia molecule with antigen. This is essentially a helper T cell version of the original altered-self model first proposed by Zinkernagel and Doherty in 1974 for cytotoxic T cell recognition of viral antigens in association with MHC-encoded class I molecules. Its major conceptual problem is how an induced or stabilized alteration could be so different for lysine versus amidinated lysine as to cause a 100- to 1000-fold shift in the dose–response curve and the elicitation of distinct families of T cell clones.

It is difficult to distinguish among these three models. Each has its virtues and its drawbacks. However, if one could measure the relative affinities of the T cell receptor from a single clonal population for the two different forms of the Ia–antigen complex, i.e., E^k_β:E^k_α–moth cytochrome *c* versus E^b_β:E^k_α–moth cytochrome *c*, then a distinction between model 2A and the other two models could be made. The T cell receptor allosteric (or induced fit) model predicts that the affinity of interaction between the T cell receptor and the antigen should be different, because the form of the Ia molecule determines the affinity of the antigen-combining site or the number of such sites that are available for binding. In contrast, the Ia-antigen physical interaction models (2B and 2C) predict that in some cases the two affinities could be the same, because the affinity of the T cell receptor for the Ia–antigen complex might not be altered by the different Ia molecule–antigen interactions. Recently, it has become possible to do an experiment to test this prediction by taking advantage of the discovery that T cell antigen receptors compete for ligand in a manner analogous to hormone receptors (Ashwell *et al.*, 1986). If one increases the number of responding T cells in culture beyond a certain critical number (the transition point), the receptors bind sufficient ligand to deplete the total amount of free ligand in the system. When this happens, there is a decrease in the percentage of cells with sufficient receptors occupied to respond. This leads to a shift in the ligand dose–response curve to higher antigen concentrations in order to achieve the same percentage of the maximal response. If the same T cell population, with a constant number of receptors per cell, is assayed in this manner using two different cross-reacting ligands, the transition points will differ depending on the affinity of the receptor for the two ligands. Thus, the ratio of the two transition points gives the relative affinities of the receptor for the two ligands. When this analysis was carried out for the T cell clones capable of recognizing the moth cytochrome *c* synthetic fragment 86–90;94–103 in association with either E^k_β

:E^k_α or E^b_β:E^k_α, the affinities were found to be the same. Thus, the potency differences observed with these two Ia molecules (about 15-fold) stemmed from other effects, such as the differences in physical interaction between the antigen and the two different Ia molecules. Although these results cannot distinguish between the trimolecular complex (2B) and the altered-self (2C) models of antigen–Ia molecule physical interaction, they do eliminate the allosteric (or induced fit) model shown in Fig. 2A. Also note that in either of the two physical interaction models one cannot distinguish between a "less repulsive" and a "more attractive" form of the physical interaction to explain the difference observed with the two Ia molecules (see Parham, 1984).

Finally, a distinction between the trimolecular complex and altered self models could be made if the T cell receptor were shown to physically interact with the antigen. The trimolecular complex model predicts this interaction will occur, whereas the altered self model predicts it will not, because the T cell receptor in the latter model recognizes only an alteration in the class II molecule (Fig. 2). Recently Rao *et al.* (1984a,b) have been able to demonstrate the binding of soluble antigen to T cell clones specific for the hapten azobenzene arsonate (ABA). When mice were immunized with ABA coupled to proteins such as keyhole limpet hemocyanin (KLH) or bovine γ-globulin (BGG), most of the T cell response was directed against the hapten, as the cells could be stimulated with ABA on almost any carrier molecule (Rao *et al.*, 1984c). T cell clones derived from long-term lines stimulated *in vitro* with ABA on different carriers (KLH then BGG) were all shown to be hapten specific. Rao *et al.* (1984a) studied the binding of ^{125}I-labeled ABA_{6-10}-OVA to several of these hapten-specific T cell clones and showed saturable binding that was specific for this type of clone and this antigen. Competition studies using analogs of ABA coupled to OVA showed an interesting result. There was a correlation between the ability of an analog to inhibit the binding of ^{125}I-labeled ABA-OVA and the ability of this analog to stimulate the T cell clone in the presence of the right Ia molecule. Any analog that could not block binding did not stimulate. However, those analogs that did block did not show a quantitative correlation with with their potency for activation. For example, the benzenesulfonate derivative coupled to OVA competed as effectively as unlabeled ABA-OVA for binding, but for activation it was about 200-fold less potent. This dichotomy between binding and activation presumably represented the contribution of the Ia molecule to the specificity of the response (as detailed in the cytochrome *c* system), although the authors preferred another interpretation.

This is one of the first reported demonstrations of the direct binding of antigen to an antigen-specific helper T cell. Another is the studies of Carel *et al.* (1983) who isolated a T cell hybridoma based on its ability to bind 2,4-

dinitroaminophenyl-derivatized cytochrome *c* fragment 66–80. Presumably these two groups succeeded where others have failed, because of the multimeric nature of the antigen (ABA_{6-10}) and/or the large portion of the binding energy directed against the hapten. However, there are still some puzzling aspects to the data. For example, in the case of Carel *et al.*, the antigen stimulated IL-2 release from the T cell hybridoma in the absence of any presenting cells. Thus, the relationship between this hybrid and conventional MHC-restricted T cells is not clear. In the experiments of Rao *et al.*, binding at saturation led to a minimum estimate of the number of receptors on the cell surface of $2–4\times10^5$, 5- to 10-fold higher than what has been estimated using monoclonal antibodies specific for the receptor (Haskins *et al.*, 1983; Meuer *et al.*, 1983; Samelson *et al.*, 1983). In addition, the dissociation curves appeared to be biphasic, with a rapid release (minutes) of 25% of the bound antigen followed by an almost undetectable release of the remaining material over several hours. Perhaps these two observations are related, in that the T cell might have two types of binding sites, one being the antigen-specific receptor and the other a nonimmunologically relevant site of significantly greater number (4-fold) and higher affinity. Nonetheless, the specificity controls and the correlation between binding and activation suggest that the antigen-specific T cell receptors on these clones are recognizing the antigen. Overall, these results argue strongly against the altered self model shown in Fig. 2 and thus leave the trimolecular complex model as the most likely physical explanation for how the Ia molecule, the T cell receptor, and the antigen interact during T cell activation.

Because the two subsites on the cytochrome *c* peptides appeared to be functionally independent of one another, a nomenclature was proposed to facilitate thinking about the separate interactions that appear to occur in a trimolecular complex model (Fig. 3). The name "agretope" was proposed for the site on the antigen that physically interacts with the Ia molecule without influencing the T cell receptor. The complementary site on the Ia molecule that interacts with the "agretope" was designated the "desetope." The part of the Ia molecule that interacts with the T cell receptor was called the "histotope" and its complementary site on the receptor, the "restitope." Finally, the site on the antigen that contacts the T cell receptor was designated the "epitope" and its complementary site on the receptor, the "paratope," in analogy to similar sites in an antibody–antigen interaction.

The physical sizes of these sites are not necessarily identical and may be as small as a single bond from a single residue. It is also not clear at the present time whether a given site is always fixed in its biologic function. It is possible, for example, that a sequence that forms an agretope might function as an epitope in the context of a different T cell receptor and a different Ia molecule. At the present time only the pigeon cytochrome *c* system has been

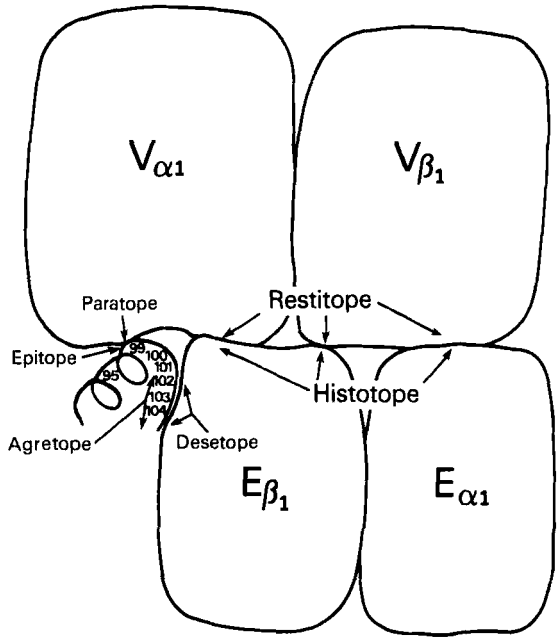


FIG. 3. Nomenclature for the contact areas in a trimolecular complex model. In the trimolecular complex model one can define three types of bimolecular interactions: (1) the T cell receptor and Ia molecule interaction, (2) the T cell receptor and antigen interaction, and (3) the Ia molecule and antigen interaction. The site on the antigen that contacts the T cell receptor is called the epitope while the site on the antigen that contacts the Ia molecule is called the agretope. The site on the Ia molecule that contacts the antigen is called the desetope while the site(s) on the Ia molecule that contacts the T cell receptor is called the histotope. The site on the T cell receptor that contacts the antigen is called the paratope while the site(s) on the receptor that contacts the Ia molecule is called the restitope. In this figure the antigen shown is the C-terminal peptide of pigeon cytochrome *c*. To date only residue 99 has been shown to contribute to the epitope and residue 103 to the agretope (Hansburg *et al.*, 1983a; Schwartz *et al.*, 1985). However, other C-terminal residues are likely to also be part of the agretope.

defined in enough molecular detail to make this nomenclature useful. I think its general value will become apparent when other antigen systems reveal similar structural features upon closer scrutiny.

d. Competitive Inhibition Experiments. If there exist two independent sites on the antigen, agretope and epitope, both necessary for T cell activation, but only one of which interacts with the Ia molecule, then it should be possible to competitively inhibit T cell activation with analogs of the antigen that share agretopes and not epitopes (for a review see R. Schwartz, 1985). For example, the amidinated derivative of pigeon cytochrome *c* fragment 81–104 should competitively inhibit the native pigeon fragment from stim-

ulating T cell clones specific for the latter. Although the predicted result was not seen in this particular experiment, several other laboratories, using different antigen systems, have been successful (Werdelin, 1982; Rock and Benacerraf, 1983, 1984a; Godfrey *et al.*, 1984). Strain 2 guinea pigs are responders to both the DNP-PLL and the GL copolymers, but the T cells elicited by each antigen are poorly cross-reactive. Werdelin (1982) showed that when T cells specific for DNP-PLL were stimulated with strain 2 macrophages pulsed with DNP-PLL, the stimulation could be blocked by exposing the macrophages to GL at the time of antigen pulsing. This inhibition was specific in that GL did not inhibit the stimulation of ovalbumin-specific T cells. In addition, other positively charged polymers such as poly-L-arginine and poly-D-lysine had no effect on the stimulation of the DNP-PLL-specific T cells. The only other successful competitor was poly-L-lysine without DNP. Interestingly, the minimal size of the polymer required to produce detectable inhibition was 8 to 12 lysine residues, an observation that correlated with the original studies of Schlossman and Levin (1971) on the minimum-sized DNP-oligolysine required for immunogenicity in guinea pigs. The reciprocal experiment, using T cells primed to GL and competition with DNP-PLL, was inconclusive as the partial inhibition observed could be attributed to the cellular toxicity of the DNP-PLL at high doses (>20 μg/ml).

The GL inhibition of DNP-PLL stimulation required that the macrophages be exposed to the GL prior to or during the pulsing with DNP-PLL. Once the cells had seen DNP-PLL, the GL would no longer inhibit. In addition, preexposure of the macrophages to GL did not inhibit the uptake of DNP-PLL by the macrophages. Overall the experiments were interpreted to mean that GL competed with DNP-PLL by binding to the strain 2 Ia molecule. Note that this binding must have been of relatively high affinity, because washing of the cells after preexposure to GL did not prevent the inhibition of DNP-PLL-induced stimulation. Furthermore, the turnover of Ia molecules was not considered to be a problem, because the initial association with antigen was postulated to occur inside the cell after antigen processing. Thus, even when the inhibitor was used in the prepulsing experiments, enough remained inside the cell to compete for association with newly synthesized Ia molecules.

These observations were confirmed and extended by Rock and Benacerraf (1983). Using T cell hybridomas specific for GAT, they showed that hybrids recognizing the antigen in association with A^d_β:A^d_α could be inhibited from releasing IL-2 if the nonstimulatory GT copolymer was also added to the cultures. The GT copolymer did not inhibit hybrids recognizing GAT in association with A^b_β:A^b_α, even if the same $(b \times d)F_1$ presenting cells were used to present the antigen. The inhibition was competitive, could be carried out

during the antigen-pulsing step, and required that the GAT and GT be added to the same presenting cells in culture. The best control was a T cell hybridoma with dual reactivity. It recognized GAT in association with A^d_β:A^d_α and Ia^d alone (autoreactivity). GT only inhibited the IL-2 release induced by the antigen in association with A^d_β:A^d_α and not the autoreactivity. If the same T cell receptor was responsible for recognition of both determinants, then the selective inhibition by GT could not be at the level of the T cell receptor, but had to be at the level of the Ia molecule–antigen interaction.

Rock and Benacerraf (1984a,b) extended their observations to another antigen–Ia molecule system, the GLPhe copolymer in association with E^d_β:E^d_α inhibited by poly$(Glu^{62},Phe^{38})_n$ and poly$(Glu^{55},Lys^{35},Leu^{10})_n$, and also explored the overlap of self Ia molecule and antigen recognition with recognition of allogeneic Ia molecules. In the latter case, Rock and Benacerraf (1984b) reasoned that allo-recognition of polymorphic determinants on Ia molecules must sometimes involve the determinants used as desetopes in the Ia molecule–antigen interaction (see Fig. 3). Therefore, they predicted that some alloreactive T cell hybridomas should be inhibitable by antigens that use the same Ia molecule as a restriction element. On screening a panel of C57BL/10 (H-2^b) anti-BALB/c (H-2^d) T cell hybrids, they found 10% (7/73) that were inhibitable with GAT. The inhibition was dose dependent and correlated with the GAT concentrations required to stimulate a response from other hybrids specific for GAT in association with A^d_β:A^d_α. In addition, the GT copolymer also inhibited these alloreactive clones and inhibition was seen if the presenting cells were prepulsed with the antigens. These results suggested that the polymers were blocking the allodeterminant by binding to it (or near it) and preventing the T cell receptor from recognizing it.

Finally, Godfrey *et al.* (1984) have recently shown that analogs of even the small antigen, tyrosyl azobenzene arsonate (ABA-Tyr), could competitively inhibit T cell activation. In these experiments, the proliferation of normal T cell clones specific for ABA-Tyr was selectively blocked by the azobenzene derivative of tyrosine (PAPA), a nonstimulatory analog of the molecule. Again, prepulsing experiments suggested that the site of blocking was at the level of the antigen-presenting cell. Overall, these observations strongly support the concept that the antigen and the Ia molecule physically interact and thus are consistent with an antigen-presentation model of *Ir* gene function. But perhaps more importantly, these experiments suggest that the antigen and the Ia molecule physically interact even in the absence of the T cell receptor. This is a necessary prediction of the altered self model shown in Fig. 2C, but it is also consistent with the trimolecular complex model shown in Fig. 2B. The quantitative aspects of the blocking suggest that the interaction is of high affinity and therefore should be detectable by direct binding studies.

e. Macrophage Factors. So far, direct evidence for a physical association between Ia molecules and antigen has only been demonstrated with factors derived from macrophages. Erb and Feldman (1975) discovered that macrophages could facilitate antigen-specific T helper cell induction across a nucleopore membrane, suggesting the existence of a soluble factor. This factor could be obtained from supernatants of purified macrophages cultured with antigen for 4 days. The action of the factor was antigen-specific and genetically restricted, i.e., supernatants from allogeneic macrophages did not work. Genetic mapping studies demonstrated that compatibility at the *I-A* subregion between the responding T cells and the macrophage donors of the supernatant was required to induce keyhole limpet hemocyanin (KLH)-specific helper T cells. Finally, supernatants obtained from antigen-pulsed nonresponder macrophages would not facilitate helper cell induction in a (responder×nonresponder)F_1 T cell population.

Biochemical characterization of the macrophage factor using column chromatography demonstrated an apparent molecular mass of approximately 55,000 Da. The factor bound to and could be eluted from Sepharose columns coated with either anti-Ia or anti-antigen antibodies, suggesting that the factor was composed of both antigen and Ia molecules. It did not bind to columns coated with anti-immunoglobulin antibodies. Because the size of these factors was relatively small even for antigens as large as KLH (subunit size 90,000 Da) it was inferred that the antigen was present in the factor in a fragmented form. Using ^{125}-I-labeled antigens, Puri and Lonai (1980) demonstrated direct binding of macrophage-processed antigen to Lyt 1^+2^- T cells by autoradiographic techniques, and selective suicide of helper activity by prolonged exposure to the radioactive antigen. In addition, the processed antigen was 100- to 1000-fold more immunogenic than regular antigen. These results suggested that the antigen–Ia molecule complex might be the functional biochemical unit that the macrophage uses to stimulate T cells. However, so far, the nature of the chemical interaction between the antigen and the Ia molecule has not been elucidated and no evidence has been reported to show that such complexes do not form between antigen and nonresponder Ia molecules. Furthermore, although the purified complexes could stimulate helper activity, they were totally incapable of stimulating an antigen-specific, MHC-restricted T cell proliferative response (Schwartz and Erb, unpublished observations). Thus, a number of hurdles remain to be surmounted before these factors can be used as evidence in favor of an antigen-presentation model of *Ir* gene function.

f. Antigen Presentation by Macrophages from Nonresponder Animals. Although, as mentioned earlier, recent studies on T cell clones have presented some of the strongest evidence in favor of antigen-presentation models, these tools have also provided the basis for experiments that represent some

of the strongest challenges to the model. Clark and Shevach (1982) isolated T cell colonies from responder strain 2 guinea pigs that recognized the antigen, GL, in association with nonresponder strain 13 Ia molecules. These colonies were derived from primary cultures in which strain 2 T cells were stimulated with irradiated strain 13 macrophages pulsed with GL. After 14 days the bulk cultures were harvested and the cells cloned in soft agar. Colonies were picked and the cells tested both for alloreactivity against strain 13 macrophages alone and antigen reactivity in the presence of strain 13 macrophages. Most of the colonies contained T cells that were alloreactive, responding to strain 13 macrophages with or without antigen. However, a minority of colonies contained T cells that only proliferated in the presence of strain 13 macrophages and antigen. The cells did not respond to antigen in association with syngeneic strain 2 macrophages and the proliferative response was only blocked by anti-Ia antibodies directed against strain 13 Ia molecules. Thus, the T cells were specific for the antigen in association with the allogeneic, nonresponder Ia molecule. These experimental results were interpreted to mean that the nonresponder macrophages were fully capable of antigen presentation, thus arguing against a defect in nonresponder Ia molecule–antigen (GL) interaction.

Similar clones have been isolated from (responder×nonresponder)F_1 murine T cell lines by Kimoto *et al.* (1981). They immunized (B6×A/J)F_1 mice with (T,G)-A--L and set up long-term T cell lines *in vitro* by repeated stimulation with antigen and (B6×A)F_1 spleen cells. Analysis of these lines for MHC restriction revealed that after 5 months, one of them became dominated by T cells specific for (T,G)-A--L in association with nonresponder strain A/J spleen cells. Clones derived from the line showed the same property. Thus, a nonresponder Ia molecule was again shown to be capable of interacting with antigen in a functionally meaningful way. Note, however, that the origin of these clones is not clear. They could either be rare clones present in the F_1 T cell population that by chance happened to come to dominate one of the lines, or they could represent somatic variants that arose in culture following the multiple rounds of antigen-driven division and selection that these lines undergo. In either case, they would appear to represent an unusual situation, because freshly primed (B6×A)F_1 T cells do not proliferate significantly in response to (T,G)-A--L in association with nonresponder strain A/J presenting cells (R. Schwartz *et al.*, 1977).

The existence of these clones argues strongly against an antigen-presentation model in its absolute form, i.e., where antigen is postulated not to bind to nonresponder Ia molecules at all. In such an extreme case, no T cell clone, no matter how rare or what the binding characteristics of its receptor, should be stimulated by the combination of nonresponder Ia molecule and antigen. Thus, the fact that such clones exist disproves the absolute

form of the model. However, biologically it is obvious that *Ir* gene control is not absolute. In many circumstances the nonresponder strain can be shown to make a small response when the immunization dose is increased. Similarly, in physicochemical terms, any two molecules put together in solution or on the surface of a membrane at an appropriate distance from one another will always interact in some way, whether it be a repulsive or an attractive interaction. Therefore, it seems likely that the more relevant way to consider an antigen-presentation model is in quantitative rather than qualitative terms. In this context the relevant *Ir* gene question becomes what is the difference in the binding constants that describe the interaction of the antigen with the Ia molecules from the responder and nonresponder strains? In this kind of quantitative model, it is perfectly conceivable that a high affinity T cell clone might be found that could be activated by the antigen in association with the nonresponder Ia molecule. Thus, the relevant Ir question now becomes, why is the frequency of such clones so rare in the nonresponder? If this is because the interaction of the Ia molecule and the antigen is so unfavorable that only a few clones of T cells possess a high enough affinity to stabilize the interaction, then at least some form of an antigen-presentation model is viable. In contrast, if such clones normally exist in large numbers in strains other than the nonresponder, then models postulating T cell repertoire constraints would seem to be a more likely explanation of nonresponsiveness.

Ishii *et al.* (1982a,b) made an attempt to distinguish between these two possibilities by undertaking an extensive series of experiments to identify T cells capable of recognizing antigen in association with nonresponder Ia molecules. They developed an *in vitro* system for priming proliferative T cells to soluble antigens, such as poly$(Glu^{60},Ala^{40})_n$ (GA), and then determined the specificity of the T cells in a secondary proliferation assay. This system yielded results consistent with previous *in vivo* priming protocols. Responder T cells could be primed to antigen with syngeneic antigen-presenting cells, whereas nonresponder T cells could not. (Responder $\times$nonresponder)F_1 T cells primed with antigen on F_1 or responder antigen-presenting cells manifested a large proliferative response when rechallenged with antigen and F_1 or responder antigen-presenting cells. In contrast, nonresponder antigen-presenting cells would not prime the F_1 T cells.

In order to determine whether the F_1 T cell population did not respond to antigen in association with nonresponder Ia molecules because the F_1 lacked T cell clones with this specificity, or because of a failure of the antigen to interact well with nonresponder Ia molecules, they looked in allogeneic strains for T cells capable of recognizing the antigen in association with nonresponder Ia molecules. In order to test for the presence of such clones, they had to first deplete the T cell populations of lymphocytes that were

alloreactive to the MHC-encoded molecules of the nonresponder haplotype. This was accomplished by exposing the population first to nonresponder antigen-presenting cells alone and then eliminating the dividing cells by the introduction of bromodeoxyuridine and light. Subsequent to this elimination, the remaining T cells were again stimulated with the allogeneic nonresponder antigen-presenting cells, this time in the presence of antigen. The T cells were then assayed for priming in the secondary proliferation assay. Surprisingly, all but one allogeneic T cell population contained clones capable of recognizing the antigen, GA, in association with the nonresponder Ia molecule. The source of the T cells could be strains that responded to GA, such as C57BL/10 and BALB/c, or even more amazingly, strains that were themselves nonresponders to the antigen, such as B10.P, A.SW, and DBA/1. Thus, although a nonresponder strain did not possess many clones of T cells capable of responding to the antigen in association with its own Ia molecules, it did appear to contain T cell clones capable of recognizing the antigen in association with Ia molecules from other nonresponders. This suggested that any given nonresponder is only blind to the antigen in association with its own Ia molecules, an observation more compatible with a repertoire deletion model (discussed in the next section) than with an antigen-presentation model. Overall, the data suggested that the existence of clones specific for the antigen in association with nonresponder Ia molecules was very common.

However, the fact that almost all allogeneic strain combinations seemed to possess such clones raised the possibility of some kind of experimental artifact. It is likely that the T cells with this specificity do exist, because the cloning experiments discussed above have clearly isolated such cells. The critical question is whether these clones are rare and have been amplified in the *in vitro* primary and secondary stimulations by residual alloreactive cells. Bromodeoxyuridine and light treatment is the least effective of all the acute depletion protocols, removing only 80–90% of the alloreactive T cells (Maryanski *et al.*, 1980). Thus, it is possible that this is an inadequate depletion and that the remaining allogeneic cells, when reexposed to the alloantigens, release sufficient lymphokines to support the expansion of the small number of antigen-specific clones, even though the allogeneic cells themselves do not undergo much detectable proliferation. This could come about if the affinity of the remaining cells for alloantigen were too low to drive the cells into S phase in the cell cycle, but high enough to result in lymphokine release.

The solution to this potential criticism is to examine T cell populations depleted of alloreactive cells by other methods. The results with radiation-induced bone marrow chimeras (discussed in Sections III,E and V,B,1) contradict the bromodeoxyuridine and light experiments. However, one

could rightly argue that in this case the T cell repertoire completely redevelops in the new environment and thus deletions in the repertoire could easily occur. What one needs is other methods of acute depletion. So far, no experiments have been done with *in vivo* filtration and thoracic duct drainage. However, one experiment has been reported by Kohno and Berzofsky (1982) in which neonatal tolerance was used as the mechanism of depletion. In this study, low responder B10.BR mice were neonatally tolerized to high responder B10.D2 alloantigens by iv injection of (B10.BR×B10.D2)F_1 spleen cells, and high responder B10.D2 mice were tolerized to low responder B10.BR or B10 alloantigens by injection of (B10.BR×B10.D2) or (B10×B10.D2)F_1 spleen cells. At 8 weeks of age the mice were primed with myoglobin and their T cells examined for helper activity with high or low responder B cells. Neonatally tolerant low responder B10.BR mice possessed a population of T cells capable of recognizing the antigen in association with high responder B cells, but neonatally tolerant high responder B10.D2 mice did *not* possess a population of T cells capable of recognizing the antigen in association with low reponder B cells. Elimination of residual F_1 tolerizing cells with anti-H-2 antibodies and complement did not alter these results. Thus, in this one experiment using a different protocol for depletion of alloreactive cells, the results of Ishii *et al.* (1982a,b) were not substantiated. On the other hand, numerous differences exist between the two sets of experiments, such as the antigens and the assay systems that were utilized. Even more important is the possibility that neonatal tolerance might delete the clones being sought because these clones are part of the alloreactive pool, or because they are cross-reactive with self antigens in association with the allogeneic Ia molecules (see Section VI,C,2). Clearly, many more comparisons need to be done in this area and, if possible, a limiting dilution assay set up for determining the frequency of cells with a given phenotype in the population, before and after acute depletion. Only after such quantitative comparisons have been made will it be possible to determine whether the existence of T cells specific for antigen in association with nonresponder Ia molecules represents a serious challenge to antigen-presentation models of *Ir* gene function.

C. Models Involving Constraints on the T Cell Repertoire

1. Limitations in the Germline Repertoire

a. Structure of the Antigen-Specific T Cell Receptor. In considering the failure of a given population of T cells to respond to a particular combination of antigen and Ia molecule, proponents of the type of models to be discussed in this section would suggest that the T cell repertoire is lacking clones with receptors specific for this combination. The differences between the models

revolve around the mechanisms by which the constraints are placed on the T cell repertoire to generate these so called "holes" in the recognition network. We will discuss four of these models in detail.

The antigen-specific receptor on helper and cytotoxic T lymphocytes has been only recently characterized. The development of T cell cloning technology in the late 1970s (reviewed in Möller, 1981) paved the way for the production of antireceptor monoclonal antibodies (Allison *et al.*, 1982; Haskins *et al.*, 1983; Meuer *et al.*, 1983; Samelson *et al.*, 1983), which were used to isolate and biochemically characterize the molecules (reviewed in Möller, 1984). In addition, these clonal populations were used to produce T cell-specific cDNA libraries by subtractive hybridization (Hedrick *et al.*, 1984). From these libraries the genes encoding the receptor molecules were isolated (Davis *et al.*, 1984; Mak and Yanagi, 1984; Chien *et al.*, 1984; Saito *et al.*, 1984). The present consensus on the structure of the T cell antigen receptor is that it is composed of two polypeptide chains, α and β. Each chain is a transmembrane glycoprotein of approximately 40,000–50,000 Da containing one variable region and one constant region domain, each containing one intrachain disulfide bond. In the mouse, the α chain has an acidic p*I*, a core size of 28,000 Da, and 4 N-linked carbohydrate side chains. The β chain has a neutral to slightly basic p*I*, a core size of 31–32,000 Da, and 3 N-linked carbohydrate side chains. The α and β chains are covalently linked by a disulfide bond and expressed on the cell surface as an 85–90,000 Da heterodimer.

At the DNA level, only the genomic organization of the β chain gene has been completely analyzed. It is composed of a family of *V* region exons, each with its own leader exon, two families of diversity (*D*) exons, two families of joining (*J*) exons, and two constant region exon sets, each composed of a *C* exon, a small 18 base pair exon encoding the region around the interchain disulfide bond, a transmembrane (*TM*) exon, and an intracytoplasmic (*IC*) exon. The two *J* families each contain 6 functional *J* elements; the $D_{\beta 2}$ "family" appears to contain only one element and the extent of the $D_{\beta 1}$ family has not been fully characterized. The β chain *V* region genes are significantly different from immunoglobulin *V* region genes (Patten *et al.*, 1984). They show extensive sequence diversity, one from another, and thus comprise small families of only one or two elements. In addition, this diversity extends across mammalian species, as evidenced by a lack of extensive cross-hybridization, implying that rapid evolutionary diversity of these genes is an ongoing phenomenon. The total size of the β chain *V* region gene pool appears to be small as evidenced by the high frequency with which each *V* region gene is expressed in cDNA libraries. In fact the same *V* region gene has been found in use in a pigeon cytochrome *c*-specific, E^k_β:E^k_α-restricted T cell hybridoma and a hen egg lysozyme-specific A^b_β:A^b_α-restricted T cell clone

(Goverman *et al.*, 1985). Even more surprisingly, the same V_β exon has been found in use in a helper T cell clone specific for chicken red blood cells in association with A^b_β:A^b_α (Patten *et al.*, 1984) and an alloreactive cytotoxic T cell clone specific for D^d (V. D. Starz, M. J. Bevan, and S. M. Hedrick, personal communication).

The pattern of diversity of the *V* region genes of the α chain is different. Although genomic clones are not yet available, Southern blot analysis using *V* region probes showed cross-hybridization to a large number of bands (6–8), suggesting bigger gene family sizes more akin to those of immunoglobulin heavy chain *V* region gene families (Chien *et al.*, 1984; Saito *et al.*, 1984). *J* elements could also be discerned in the sequences of the cDNA clones. The possibility of *D* elements was also suggested.

The normal generation of somatic diversity in the β chain of the receptor appears to occur entirely during the recombinational events that link the *V*, *D*, and *J* elements together. So far, the expressed *V* region genes that have been sequenced at both the cDNA and genomic levels have been identical, suggesting no somatic hypermutation mechanism after exposure to antigen.[3] Instead, the flanking sequences around the *V*, *D* and *J* elements, which presumably serve as recombination-enzyme recognition sites, are ordered in such a way that one *D* element could join to another *D* element, in addition to *D–J* joining. This creates the potential for more combinatorial diversity than one finds in immunoglobulin heavy chain genes. Whether N-type diversity (Alt and Baltimore, 1982), caused by terminal deoxynucleotidyltransferase (TdT), takes place at *V–D* and *D–J* junctions is not clear, because all the *D* elements have not been sequenced. However, this is likely to be a major mechanism of diversity since the thymus is the principal organ from which this enzyme was isolated. The mechanisms generating diversity in the α chain of the receptor are unknown at the present time.

b. Repertoire Diversity. From this brief overview of T cell receptor structure and genetic assembly, it is clear that a significant level of clonal diversity could potentially exist in the T cell repertoire. The combinatorial diversity of the β chain coupled with the probable V region diversity of the α chain, should yield as much clonal diversity in T cell receptors as is generated in immunoglobulin molecules during B cell development (prior to the introduction of antigen). However, whether this would be sufficient to recognize

[3] Recently Sim and Augustine (1984) have isolated a somatic T cell hybridoma that appears to give rise to progeny with mutations in the antigen-specific receptor. The relevance of this clone to the normal generation of receptor diversity is not clear at the present time. Similar to the S107 myeloma studied by Cook and Scharff (1977) for immunoglobulin receptor diversity, this T cell hybridoma is unique in its phenotype. Perhaps it represents a dedifferentiated cell that could give us insight into the mechanism of the generation of receptor diversity in immature T cells.

all combinations of foreign antigenic determinants and self MHC-encoded molecules is not certain. Immunoglobulin receptors generated during ontogeny are clearly not sufficient to recognize all foreign antigens with high enough affinity (see discussion in Cohn *et al.*, 1974). Hence the need for somatic hypermutation after the introduction of antigen. However, T cell receptors might not require high affinity interactions in order to function, possibly because the antigen is presented in a multimeric form on the surface of another cell (see discussion in Schwartz, 1985). In this regard, it is intriguing that the same β chain *V* region gene is employed by both a cytotoxic and a helper T cell clone of different specificities. One might have thought that class I and class II molecules would be sufficiently different in structure to require distinct V regions for recognition. Although it is still possible that the α chain V region differences will be responsible for most of the specificity, the observation suggests the possibility that the T cell receptor functions via a relatively low affinity interaction with its ligands. If so then the number of specificities generated by recombinational and junctional (including N type) diversity acting on a modest number of germline *V*, *D*, and *J* elements might be sufficient to ensure a totipotent T cell repertoire.

On the other hand, if this degree of diversity is not sufficient to generate a totipotent repertoire, then holes could exist that would potentially account for some forms of *Ir* gene defects. If recognition of foreign antigen in association with a self MHC-encoded molecule occurs via a T cell receptor with a single combining site formed by the interaction of the V region domains of the α and β chains (in a manner analogous to the interaction between the V region domains of heavy and light chains), then a limited germline repertoire might preclude the formation of a combining site with high enough affinity for the antigen in association with one allelic form of the histocompatibility molecule but not another. In such a case, all strains (individuals) in the species could lack the appropriate *V*, *D*, and/or *J* elements, but only the strain expressing one particular allelic form of the histocompatibility molecule would manifest the *Ir* gene defect. If, in addition, differences in *V* region genes exist between strains, one would expect to find examples of *Ir* genes mapping to chromosome 6 (β chain locus) or chromosome 14 (α chain locus) in the mouse. To date no such *Ir* genes have been detected. However, polymorphisms have been shown to exist at these loci (Haskins *et al.*, 1984). Therefore, perhaps when congenic strains are derived, which differ at these loci but not at *H-2*, it will be possible to identify V_T-linked *Ir* genes by immunizing the animals with antigens containing single T cell determinants. How important such germline mechanisms will be in causing *Ir* gene defects in the animal will have to await a more detailed study of the diversity of the β and α chain variable regions.

2. *Tolerance Models*

*a. The Early Model: Self MHC = X.*Tolerance models are the oldest of the somatic T cell-depletion mechanisms proposed to explain *Ir* gene function (Jenkin, 1963; Cinader, 1963). Because the immune system is capable of recognizing almost any foreign antigen it encounters, it was long felt that the failure to react against self antigens must be an acquired trait. With the demonstration by Billingham *et al.* (1954) that the development of an immune response to histocompatibility antigens could be prevented by exposure to these antigens *in utero,* the idea of immunological tolerance as the mechanism for nonresponsiveness became one of the dominant theories in immunology. It was thus natural when *Ir* genes were discovered to try and explain their function within the basic theoretical framework of tolerance (see Snell, 1968, for a discussion). *Ir* genes map to the MHC and lead to selective defects in the ability to respond to specific antigens. Therefore, it was proposed by Cinader (1963) that certain foreign antigens (such as a virus or a synthetic polypeptide) must structurally resemble particular MHC-encoded gene products (i.e., self MHC=X). Thus, when self-tolerance to these major histocompatibility antigens was imposed, immune cells capable of recognizing the foreign antigen were also eliminated. Responder animals would consist of those individuals whose MHC-encoded molecules were sufficiently different in structure such that T cell clones capable of recognizing the foreign antigen were not eliminated.

However, this type of molecular mimicry model has a number of problems (see Benacerraf and McDevitt, 1972, for a discussion). First is the observed phenotype of the F_1 cross between a responder and a nonresponder. A tolerance model would predict that nonresponsiveness should be dominant since the nonresponder MHC-encoded molecules are expressed in the F_1 and therefore should eliminate the immune cells specific for the foreign antigen. In fact, as discussed earlier in this review (Section II,A), responsiveness has been shown to be dominant or codominant in numerous antigenic systems. Second, an exhaustive search by McDevitt (1968) in two *Ir* gene-controlled systems, the response to (T,G)-A--L and (H,G)-A--L in *H*-2^b and *H*-2^k mice, failed to find any evidence for immunologic cross-reactivity between these synthetic polypeptides and the low responder allelic form of the histocompatibility molecule, at either the antibody (B cell) or cross-priming (T cell) levels. Finally, it is conceptually difficult to imagine how tolerance to a few histocompatibility proteins (e.g., the one molecule encoded by the A^b_β and A^b_α genes) could eliminate responses to a large number of other families of antigens [e.g., (H,G)-A--L, pigeon cytochrome *c*, GLPhe, etc.]. This could be possible only if the universe of recognizable antigenic configurations was small, a situation that seemed implausible given

the specificity and diversity of the immune system. For these reasons, tolerance models were eclipsed from immunological thinking for a number of years.

b. The MHC-Restricted Tolerance Model. With the discovery of MHC restriction an interest in tolerance models was rekindled. Now it was evident that the T cell receptor did not recognize antigen alone. Thus, if one chose to interpret MHC restriction in terms of a single T cell combining site for antigen and the histocompatibility molecule, then most of the objections to the idea of tolerance as an explanation for *Ir* gene function disappeared. In the first model of this type, proposed by R. Schwartz (1978) it was postulated that self MHC-encoded molecules seen during ontogeny in association with other self antigens would mimic the structures formed by self MHC-encoded molecules seen in association with foreign antigens, i.e., self Ia molecule plus self antigen X = self Ia molecule plus foreign antigen Y. If tolerance induction obeyed the same rules as antigen presentation, then T cell clones capable of seeing self antigen X in association with a self Ia molecule would be eliminated, thus preventing a later response to foreign antigen Y. This proposal circumvented the devastating effects of the F_1 experiments in that it predicted that responsiveness would be codominant. For example, if strain A is a responder to antigen Y and strain B is a nonresponder, then elimination of T cell clones in the F_1 that recognize Y plus Ia^b, by cross-reactive tolerance for self antigen X plus Ia^b, should have no effect on the T cell clones that see the same foreign antigen Y in association with the responder Ia^a. The F_1 is a responder because it retains clones that see Y plus Ia^a, not because it acquires the ability to recognize Y plus Ia^b. This prediction was confirmed by experiments with radiation-induced bone marrow chimeras of the type nonresponder parent(b.m.) → (responder×nonresponder)F_1(irr). As discussed earlier (Section III,E), when the nonresponder T cells acquire responsiveness to the antigen by developing in a high responder environment, the recognition potential they achieve is only for antigen in association with the responder Ia molecules. T cells recognizing the antigen in association with nonresponder Ia molecules were not found.

The MHC-restricted tolerance model also explained why the immune responses to (T,G)-A--L and (H,G)-A--L did not show cross-reactivity on nonresponder histocompatibility antigens and the immune responses to the histocompatibility antigens did not show cross-reactivity on (T,G)-A--L and (H,G)-A--L. These two sets of antigens alone are not the structures that mimic each other. To search for immunologic cross-reactions to test the newer model, one would have to know the nature of the self antigen involved in the tolerance induction. Finally, the model also satisfactorily overcame the problem of the paucity of tolerogenic determinants on MHC-encoded molecules, as now numerous self antigens of widely different structures were also part of the tolerance-inducing complex.

c. Alloreactivity of Antigen-Specific Clones. Evidence to uniquely support a tolerance model of *Ir* gene function is not extensive. Experiments from several laboratories (C. Pierce *et al.*, 1976; C. Pierce and Kapp, 1978a; S. Pierce *et al.*, 1980; Speck *et al.*, 1981) demonstrated that responder spleen cell populations contain T cells capable of helping B cells from nonresponder mice to make an antibody response. In two of these cases it was also shown that the (responder×nonresponder)F_1 population lacked such T cells. These results suggested that T cells with receptors specific for antigen in association with nonresponder Ia molecules did exist, but that they were deleted in the F_1 and nonresponder strains by a tolerance mechanism. However, this interpretation was challenged by critics who argued that the presence of alloreactive T cells in the responder, but not the F_1, populations led to allogeneic effects that by-passed normal *Ir* gene control (Janeway, 1983). For example, allostimulation could lead to the production of lymphokines such as B cell growth factor and B cell differentiation factor, which in consort with the antigen could activate the nonresponder B cells.

A more convincing argument for a tolerance model can be made from the recent finding that many antigen-specific T cell clones also manifest a particular alloreactivity (Von Boehmer *et al.*, 1979; Sredni and Schwartz, 1980; Braciale *et al.*, 1981b; R. Schwartz and Sredni, 1982; Janeway *et al.*, 1982). This has been shown for both proliferative and cytotoxic T cells. In one case, M. Kimoto and C. G. Fathman (personal communication) found a GAT-specific clone from a (B6×A)F_1 mouse that had alloreactivity to an Ia molecule of $H\text{-}2^s$, a low responder haplotype. Thus, one could be reasonably sure that this clone would not be found in a B10.S mouse, because this animal should be tolerant to its own histocompatibility antigens. The relevance to the B10.S mouse of a clone that sees GAT in association with (B6×A)F_1 MHC gene products is moot. However, from this observation one can extrapolate to the general case in which there will be clones in a responder strain, A, that recognize both GAT in association with MHC A-encoded Ia molecules and alloantigens on some nonresponder strain B MHC-encoded Ia molecules. Thus, when a cross is made between the A and B strains, the F_1 hybrids will have these clones deleted by a tolerance mechanism.

The best experimental example of this is actually in the cytotoxic response to the male-specific antigen, H-Y (Müllbacher *et al.*, 1981), which will be extensively discussed in Section VII,C. The relevant results for the current discussion can be briefly summarized as follows. Both B10 and (B10×B10.S)F_1 mice respond to the H-Y antigen. However, the F_1 cytotoxic response is predominantly specific for H-Y in association with $H\text{-}2^s$-encoded class I molecules. The question arose as to why the F_1 mice did not make a cytotoxic response to H-Y in association with $H\text{-}2^b$-encoded class I molecules. The answer, according to Müllbacher *et al.* (1981) appears to be that the B10 clones elicited in response to immunization with the H-Y antigen are dual

reactive and show allogeneic killing of B10.S targets. Such clones would be expected to be eliminated in the (B10×B10.S)F_1 in order to ensure self tolerance. An almost identical observation was subsequently made by Vučak *et al.* (1983) who found that cytotoxic T cells specific for H-Y in association with the K^{w3} allelic product were also alloreactive for D^P. These clones were not found in the H-Y response of B10.STA39, which is an MHC-congenic strain bearing K^{w3} and D^P.

A similar mechanism could also be operating in the experiments of Ishii *et al.* (1982b) described in Section VI,B,2. In particular, Ishii *et al.* (1983) claimed to have observed such a deletional process *in vitro* when they studied the GLT response of B10.A(4R) mice (normally a nonresponder strain) presented on B10.A(5R) or B10.S(9R) antigen-presenting cells. To do this they first depleted the B10.A(4R) population of T cells alloreactive to B10.A(5R) or B10.S(9R) MHC-encoded molecules using bromodeoxyuridine and light, and showed that the remaining cells could respond to GLT in association with B10.A(5R) or B10.S(9R)-presenting cells, respectively. However, in either case, if B10.A(2R) cells were also included in the initial depletion regimen with bromodeoxyuridine and light, then the remaining B10.A(4R) T cells did not respond to GLT in association with either B10.A(5R) or B10.S(9R) antigen-presenting cells. This experiment suggested that B10.A(4R) clones that are alloreactive for B10.A(2R) Ia molecules (which must be anti-E^k_β·E^a_α because of the MHC genetics of these two strains) are the same T cells that recognize GLT in association with B10.A(5R) and B10.S(9R) Ia molecules. Thus, deletion of alloreactivity *in vitro* appeared to lead to the loss of recognition of an antigen in association with another Ia molecule.

One important caveat in interpreting these experiments is whether the clones that recognize the antigen in association with the allogeneic Ia molecules derive from the residual alloreactive pool of cells not eliminated by the bromodeoxyuridine and light treatment. For example, B10.A(5R) stimulators might deplete all high affinity clones that see Ia molecules of the i5 haplotype, but leave 10% of the T cells capable of making such a response. These remaining 10% would presumably be of lower affinity and might not give a detectable proliferative response when restimulated with Ia molecules alone, because of the suboptimal culture conditions used in the experiments (the assays were done in horse serum instead of fetal calf serum). Some of the cells in this population might proliferate in response to GLT in association with B10.A(5R) Ia molecules. However, if these clones were also high affinity alloreactive for B10.A(2R) Ia molecules, then they would be eliminated when B10.A(2R) cells were added to the negative selection protocol. Thus, the responding clones would be really low affinity for an allogeneic Ia molecule and high affinity for the antigen in association with this Ia molecule. Such T cells are known to exist, as they have been cloned in other systems (Hedrick *et*

al., 1982; Matis *et al.*, 1983b). However, these clones would not be the ones found in [B10.A(4R)×B10.A(5R)]F_1 mice responding to GLT, as they would be eliminated by the more thorough tolerance induction process. This whole scenario could be examined by setting up B10.A(5R)(b.m.)→B10.A(4R)(irr) chimeras and testing them for responsiveness to GLT. The experiments of Ishii *et al.* (1983) suggest that the B10.A(4R) population contains T cell clones that recognize GLT in association with B10.A(5R) Ia molecules. These chimeras have the B10.A(4R) repertoire and B10.A(5R) antigen-presenting cells. Therefore, they should respond to GLT, unless, of course, such clones are part of the alloreactive pool recognizing B10.A(5R) Ia molecules. In this case they would be eliminated by the tolerance process.

Despite this caveat, it seems to me that the real question surrounding the experiments of Ishii *et al.* (1983) is their relevance to the mechanism of *Ir* gene control. These experiments raise the possibility that the failure of the B10.A(2R) mouse to respond to GLT stems from the deletion of "B10.A(4R)-like" clones that recognize GLT in association with B10.A(2R) Ia molecules, although this was not demonstrated experimentally. This presumably would occur because these clones have too high an affinity for E^k_β:E^a_α (now syngeneic instead of allogeneic) or E^k_β:E^a_α in association with another self protein, and are deleted by a tolerance mechanism. Although this seems like a reasonable explanation, the question is whether these cells should be considered as part of the potentially antigen-responsive repertoire, since they could never be used as such in the animal. These clones are fundamentally different from those in the responder strain. The latter do not respond to Ia molecules alone, but only to antigen in association with self Ia molecules. One presumes that the responder repertoire also originally contained a number of clones that were weakly self reactive and could recognize the antigen in association with self Ia molecules, but that these were eliminated by the tolerance mechanism. Thus, one could argue that the only legitimate clones to ask about are those which only recognize the antigen in association with the nonresponder Ia molecule and not those that also recognize the nonresponder Ia molecule alone. If so, then the mechanisms responsible for the failure to stimulate the former clones are the ones we should be trying to understand.

d. Dominant Nonresponsiveness of Non-MHC-Linked IR genes. Another way in which tolerance induction could influence the T cell repertoire is if a non-MHC-encoded self antigen, either alone or in association with self Ia molecules, were to mimic a foreign antigen in association with that or another self Ia molecule. At present no direct evidence exists to support such a mechanism. However, there does exist one genetic system that potentially could yield such evidence. This is the *Ir-2* locus described by Gasser (1967). The immune response to the murine Ea-1 erythrocyte antigen (equivalent to

the A, B, O system in man) is regulated by several genes, one of which, *Ir-2*, maps close to the *agouti* locus on the second chromosome, between the *H-3* and *H-13* histocompatibility loci. The YBR strain is a responder to Ea-1, whereas the BALB/c strain is a nonresponder. In contrast to the responsiveness of the F_1 hybrid for MHC-linked *Ir* genes, (YBR×BALB/c)F_1s were nonresponders to Ea-1. This observation of dominant nonresponsiveness by a non-MHC-linked gene is a prediction of this kind of tolerance model (see R. Schwartz, 1978). YBR and BALB/c both possess the $H\text{-}2^d$ haplotype. Therefore, T cell recognition of Ea-1 in association with $H\text{-}2^d$ gene products is influenced by one allele of the *Ir-2* locus, the $Ir\text{-}2^b$ allele of the BALB/c, and not the other, the $Ir\text{-}2^a$ allele of the YBR. If the $Ir\text{-}2^b$ allele coded for a self antigen that in association with $H\text{-}2^d$-encoded Ia molecules structurally mimicked Ea-1 plus an $H\text{-}2^d$-encoded product, whereas the $Ir\text{-}2^a$ allelic product was sufficiently different so as not to produce this mimicry, then self tolerance to the $Ir\text{-}2^b$ product would lead to nonresponsiveness to Ea-1 in both the BALB/c and (YBR×BALB/c)F_1.

Gasser (1967) originally tested for a simple tolerance model, in which Ea-1 was postulated to cross-react with self antigens of the low responder BALB/c strain or the (YBR×BALB/c)F_1 hybrid, by injecting anti-Ea-1 serum into the low responder strain to determine if that would reduce the antibody titer by absorption. In no case was any absorption observed, suggesting that the nonresponders did not possess antigens that serologically cross-reacted with Ea-1. However, a number of years later, he tried a different approach which did indicate that a tolerance mechanism was at work (Gasser, 1976). In these experiments, responder YBR mice were made tolerant to BALB/c cells by injecting YBR neonates with nonresponder (YBR×BALB/c)F_1 spleen cells. When the YBR mice matured and were immunized with Ea-1, none of them responded. This suggested that in the process of becoming tolerant to BALB/c, exposure to non-MHC-encoded BALB/c antigens or those antigens in association with $H\text{-}2^d$-encoded gene products led to the elimination of T lymphocytes that were capable of recognizing Ea-1 in association with $H\text{-}2^d$ gene products. Thus, the experiment was consistent with a tolerance model, although the cellular mechanism(s) by which neonatal tolerance took place is far from clear. It would be interesting to do a direct test of this hypothesis by isolating T cell clones from YBR mice after priming with Ea-1. If the non-MHC-encoded antigen is expressed on antigen-presenting cells, then one should be able to stimulate the clones with BALB/c spleen cells.

Very recently Vidovic' *et al.* (1985) have been able to do this experiment in another system that holds promise as a second example of clonal deletion based on recognition of a non-MHC-encoded self antigen in association with self Ia molecules. In studying the T cell proliferative response to poly-$(Glu^{50},Tyr^{50})_n$ (GT), an antigen that does not elicit a helper T cell response

in inbred mouse strains (see Section VI,C,4,d), they found several cases in which two strains sharing the same MHC haplotype differed in their immune response phenotype. Thus, DBA/2 mice were responders to GT whereas B10.D2 mice were nonresponders. Since both strains carry the $H\text{-}2^d$ haplotype, the dominant nonresponsiveness must be controlled by a non-MHC-encoded gene. Vidovic' *et al.* (1985) showed that the proliferating T cells were Lyt 1^+2^- and that they recognized GT in association with $A^d_\beta A^d_\alpha$ (based on blocking studies with monoclonal anti-Ia antibodies). They were then able to test whether T cells isolated from DBA/2 mice primed to GT could be stimulated by B10.D2 presenting cells. They could not, even though these same presenting cells could stimulate a response in the presence of the antigen (showing that the Ia molecules in the two strains were functionally equivalent). Thus, although the experiment eliminated the possibility of an antigen-presentation defect to explain the nonresponsiveness of the B10.D2, it failed to provide the long sought positive data consistent with a clonal deletion model. Mixing experiments involving responder DBA/2 T cells and nonresponder (DBA/2×B10.D2)F_1 T cells, both before (*in vivo*) and after (*in vitro*) antigen priming, also failed to provide any evidence for suppression. Therefore, to date the best explanation for these experimental results still remains a clonal deletion mechanism. However, the non-MHC-encoded self antigen responsible for the tolerance induction is not a cell surface molecule expressed on an antigen-presenting cell.

e. Tolerance Induction is MHC Restricted. One of the central predictions of this form of a tolerance model is that tolerance induction should be MHC restricted. This prediction has been recently verified by a number of laboratories. In pursuing their studies on allogeneic T cells capable of recognizing antigen in association with nonresponder Ia molecules (see Section VI,B,2,f), Dos Reis and Shevach (1983) turned to insulin as an antigen in order to be able to define precisely the antigenic determinants being recognized by syngeneic and allogeneic T cells. Strain 2 guinea pigs normally respond to beef insulin by recognizing a determinant in the alpha loop of the A chain, whereas strain 13 guinea pigs respond by recognizing a determinant on the B chain involving a histidine substitution in beef insulin for the asparagine found at residue 10 in guinea pig insulin (Thomas *et al.*, 1981). When strain 13 guinea pig lymph node cells were depleted of alloreactivity against strain 2 macrophages using bromodeoxyuridine and light and stimulated with beef insulin-pulsed strain 2 macrophages, T cell colonies were derived that responded to beef insulin in association with allogeneic strain 2 Ia molecules but not syngeneic strain 13 Ia molecules. Some of the colonies showed weak alloreactivity against strain 2 Ia molecules alone and greater stimulation in the presence of beef insulin, while others only responded when both strain 13 Ia molecules and beef insulin were present. So far these results were similar to the earlier studies with GL and

GA described in the last section. However, now one could ask whether the allogeneic T cells recognized the alpha loop on the A chain, similar to strain 2 T cells, or the His-10 on the B chain, similar to strain 13 T cells, the two syngeneic cases. The surprising answer was that neither was true. The allogeneic strain 13 T cells recognized a self determinant in association with strain 2 macrophages, i.e., guinea pig insulin stimulated these colonies as well as beef insulin. This observation has two implications. First, since strain 13 guinea pigs do not respond to guinea pig insulin when immunized with this molecule in CFA, the results demonstrate that strain 13 is tolerant to insulin only in the context of its own Ia molecules, not in the context of the allogeneic strain 2 Ia molecules. Thus, tolerance induction must be MHC restricted, at least in part. Second, since strain $(2\times13)F_1$ guinea pigs do not respond to guinea pig insulin when immunized with this molecule in CFA, the results demonstrate that the T cell clones available to the strain 13 animal for responding to insulin plus strain 2 Ia molecules are deleted from the F_1 repertoire. This presumably occurs by the tolerance mechanism.

The other laboratories that confirmed that tolerance induction was MHC restricted worked almost exclusively with cytotoxic T cells. Rammensee and Bevan (1984) performed experiments similar to Dos Reis and Shevach and isolated clones of T cells specific for minor histocompatibility antigens in association with allogeneic class I molecules (BALB.K) by first depleting the responding population (BALB) of alloreactive clones with bromodeoxyuridine and light. The existence of such clones, specific for a self minor histocompatibility antigen in association with an allogeneic class I molecule, suggests that tolerance induction is MHC restricted. However, one problem with the *in vitro* studies using T cell clones is the possibility that the specificity for self antigen plus allogeneic MHC-encoded molecules is not a normal part of the *in vivo* repertoire, i.e., that the T cells with receptors of this specificity were generated *in vitro* by somatic mutation during the cell-cloning process.

This objection does not apply, however, to the *in vivo* experiments of Groves and Singer (1983) involving radiation-induced bone marrow chimeras. C3H.SW(b.m.) $\rightarrow$ (B10$\times$B10.BR)F_1(irr) mice were set up to allow stem cells bearing one set of minor antigens (C3H) in association with one *H-2* haplotype ($H\text{-}2^b$) to develop in an environment containing another set of minor antigens (B10) in association with two *H-2* haplotypes ($H\text{-}2^b$ and $H\text{-}2^k$). This developmental environment leads to an expansion of the C3H.SW repertoire, allowing the T cells to recognize antigens in association with $H\text{-}2^k$-encoded histocompatibility molecules. The question which Groves and Singer asked was whether the chimeric T cells would recognize C3H minor antigens in association with $H\text{-}2^k$-encoded histocompatibility molecules. Since the C3H minor antigens were expressed on cells of the donor, but not

on cells of the host, these antigens were presented to the immune system only in the context of $H\text{-}2^b$-encoded histocompatibility molecules. If tolerance induction were MHC restricted, then the chimeric T cells should not be tolerant to C3H minor antigens in association with $H\text{-}2^k$-encoded histocompatibility molecules. This is what was observed, although in quantitative terms the cytotoxicity against C3H minor antigens was somewhat less than that generated by normal $(B10 \times B10.BR)F_1$ mice. These results demonstrated that at least a portion of the T cell repertoire specific for C3H minor antigens was tolerized in an MHC-restricted manner. Similar results were obtained for cytotoxic T cells by Matzinger *et al.* (1984) using another *in vivo* model, thymus-grafted chimeric mice, and for DTH T cells by Lowy *et al.* (1984).

Overall, the combination of both the *in vitro* and *in vivo* experiments demonstrated convincingly that tolerance induction is, at least in part, MHC restricted. Whether some T cells are also tolerized by direct encounter with antigen alone has not been ruled out by these studies. Thus, deletion of clones with specificity for self antigens in association with self MHC-encoded molecules could account for some forms of *Ir* gene-controlled nonresponsiveness.

f. Constraints on Tolerance Models. One of the essential features of any tolerance model is the limitations it places on the nature of the T cell receptor. A pure tolerance model requires a single receptor unit, although this receptor may have two binding sites, one for the foreign antigen, the other for the MHC-encoded gene product. The reason for this limitation lies in the results of the F_1 experiment. If the antigen-recognition component were physically independent of the self-recognition component, then random expression of the two allelic forms of each component in any cell would lead to deletion of clones seeing the antigen in association with responder MHC-encoded gene products in addition to deletion of clones seeing the antigen in association with nonresponder MHC-encoded gene products. This would result in nonresponsiveness being dominant under all conditions, which is not the case. The only way to avoid a one receptor model and still postulate a tolerance mechanism for *Ir* gene function is to place the cause of the linked recognition in the antigen-presenting cell, i.e., to postulate a presentation mechanism in addition to a tolerance mechanism. Such a hybrid *Ir* gene model would then allow T cell recognition by two independent receptors (dual recognition).

In quantitative terms it would seem that tolerance induction should be one of the major mechanisms by which Ia molecules limit the T cell repertoire. The elimination of clones capable of responding to self Ia molecules alone should remove at least 1–5% of the T cells, based on the known frequency of alloreactive clones specific for nonself MHC-encoded mole-

cules of any one foreign haplotype (Ryser and MacDonald, 1979). In addition, the clones that see self antigens in association with self Ia molecules will also be eliminated, although one should be aware of the possibility suggested by Matzinger and Bevan (1977) that this population is equivalent to the one thought to recognize self Ia molecules alone, i.e., it is difficult to distinguish self Ia molecules from self X plus self Ia molecules, because many self antigens are proteins found on the surface of antigen-presenting cells. Finally, not all tolerance induction need be MHC restricted. In some instances self antigen X may structurally mimic foreign antigen Y in association with a self Ia molecule. In this case X need not resemble Y at all, yet none of the positive aspects of MHC-restricted tolerance induction is lost, i.e. (responder×nonresponder)F_1s are still responders; there is no cross-reaction between the foreign antigen and MHC-encoded molecules; and the large diversity of non-MHC-encoded self molecules still participate in tolerance induction. Overall then, these 3 types of tolerance mechanisms represent a potent force for shaping the T cell repertoire, which could easily account for the constraints on the repertoire we term *Ir* gene effects.

One problem with this analysis, however, is why only a few examples exist of dominant nonresponsiveness in F_1 crosses. The cytotoxic examples cited above represent loss of clones specific for antigen in association with one class I allelic product, by virtue of the allogeneic cross-reactivity of this same clone for a different class I molecule encoded by the MHC genes of the other parental strain. However, if this mechanism were the sole cause of *Ir* gene defects, one would expect to see some examples for class II molecules in which (responder×nonresponder)F_1s were nonresponders. This is because there should exist cases in which the nonresponder Ia molecule, either alone or in association with non-MHC-encoded gene products, mimics the foreign antigen in association with the responder Ia molecule.

There do exist two reports in the literature of dominant nonresponsiveness in class II molecule regulated antibody responses. One of these is the antibody response to the F antigen from mouse liver (Silver and Lane, 1975). CBA mice are high responders to Type 2 F antigen while DBA/2 mice are nonresponders. The (CBA×DBA/2)F_1 is a nonresponder. However, this dominant nonresponsiveness is not caused by a tolerance mechanism, because CBA(b.m.) → (CBA×DBA/2)F_1(irr) chimeras are responders to Type 2 F antigen (Silver and Lane, 1977). These chimeras are tolerant to DBA/2 MHC-encoded alloantigens and both CBA and DBA/2 minor antigens in association with DBA/2 MHC-encoded alloantigens. Therefore, the dominant nonresponsiveness cannot result from clonal elimination by tolerance. It appears instead to involve an antigen-presentation mechanism, since the chimeras lack DBA/2 and unique F_1 presenting cells. However, this cannot be a mechanism involving failure of association between antigen and Ia

molecules (along the lines detailed earlier in Section VI,B,2,c), since failure of DBA/2 Ia molecules to associate with the antigen should not affect the association of CBA Ia molecules with the antigen. Rather it could be a failure of the chimera to stimulate MHC-restricted suppressor T cells. This possibility will be discussed in Section VI,C,4,e.

The other system displaying dominant nonresponsiveness is the antibody response to TNP-mouse serum albumin (Urba and Hildemann, 1979). In this case, B10 is a high responder strain and B10.A and B10.BR are low responder strains. The (B10×B10.BR)F_1 is a low responder. However, examination of recombinant strains between B10 and B10.A mapped the *Ir* gene to the *I-B* subregion of the mouse MHC, a subregion now thought not to exist at the DNA level (Klein *et al.*, 1981). Although not yet studied, it is fairly certain that this *Ir* gene effect will be due to a suppressor mechanism (see Section VI,C,4,e). Thus, no examples exist of elimination of clones specific for antigen in association with responder Ia molecules by virtue of cross-reactivity with nonresponder Ia molecules. It is possible that only the MHC-encoded molecules of another responder strain will be capable of eliminating these clones. Unfortunately, to date, a systematic analysis to find such situations for class II molecules has not been carried out. On the other hand, it is possible that these examples are rare because tolerance mechanisms are not the dominant force responsible for *Ir* gene control.

A final difficulty that all tolerance models have in explaining *Ir* gene function is how to account for the loss of a whole family of non-cross-reacting clones in the nonresponder. As mentioned earlier in the review (Sections II,B and VI,B,2,b) several examples exist of structurally similar antigens that stimulate immunologically distinct populations of T cell clones in responder strains, and yet the nonresponder fails to respond at all to any of the antigens. To explain this in a tolerance model one has to postulate one of two possible structural mimicry hypotheses: (1) either a single self antigen is cross-reactive with a self Ia molecule in association with a set of related foreign antigens (x_1, x_2, x_3 . . .), or (2) a set of self antigens (s_1, s_2, s_3 . . . is cross-reactive with a self Ia molecule in association with a set of related foreign antigens. It is hard to imagine such degeneracy in the mimicry when we know that the immune system of the responder strain can distinguish completely between responder Ia molecules in association with x_1 and responder Ia molecules in association with x_2. Furthermore, in the first model it is difficult to understand how a single self MHC-encoded or non-MHC-encoded antigen could mimic all the combinations of nonresponder Ia molecules in association with x_1, x_2, x_3 . . . and yet have its allelic product (as in the *Ir-2* system) not mimic any, or at least not very many, of them. In the second model, one would have to postulate a closely linked multigene family coding for s_1, s_2, s_3 . . . , because the genetic analysis of the *Ir-2* system

suggests a single locus. In this case it is even more difficult to imagine how one "allelic" family (Ir-2^a) would lead to responsiveness while another (Ir-2^b) would lead to total nonresponsiveness.

The best way to get around this problem is to postulate that the permissive allele, which allows for responsiveness, is actually a null allele, i.e., there is no functional gene. Without a functional gene there is no product, and without a product there is no T cell deletion by tolerance. To solve the problem of degeneracy in the mimicry one has to postulate an ad hoc assumption about tolerance induction, which in essence would create a unique environment for this event to occur, where low affinity cross-reacting clones would be eliminated along with clones highly specific for the self non-MHC antigen. This would place a broad enough hole in the T cell repertoire to make it appear that a family of related clones had been depleted. Alternatively, one could postulate a suppression mechanism (see Section VI,C,4) in which the suppressor cells in the nonresponder recognize a common determinant present on all the members of the antigenic family (e.g., a determinant on the PLL backbone). Thus, it is possible, by making tolerance models more complex or combining them with suppression, to get around the problem of family deletions. However, note that antigen-presentation models have no difficulty with this problem since the "agretope," shared by all members of the family, forms the basis for the unfavorable interaction with the nonresponder Ia molecule in each case.

3. *Positive T Cell Selection Models*

a. The Jerne Model. Niels Jerne (1971) was the first to perceive that the T cell repertoire might be shaped by the recognition of MHC-encoded antigens. In his model to account for this, he postulated that the germ line genes coded for lymphocyte antigen-recognition structures that were a nonoverlapping set of molecules with specificity for the different MHC-encoded molecules possessed by the species. These receptors were postulated to be randomly expressed in individual lymphocytes and only the subset of cells that expressed receptors specific for self MHC-encoded antigens was stimulated to divide in a positive selection step in the thymus. This was followed by a negative selection step in which any self reactive clones that had not undergone somatic mutation during cell division were postulated to be suppressed. Those clones that did somatically mutate were released into the periphery and formed the T cell repertoire specific for foreign antigens.

In this model Ir gene function was explained as a secondary consequence of the generation of diversity in the thymus. When a particular self receptor was chosen and mutated away from self reactivity, it could only alter its structure in a limited number of ways in a finite period of time. As a result, this set of receptors would only be able to recognize certain antigens and not others. Thus, the animal would be a nonresponder to those antigens that

could not be recognized by its mutated anti-self receptors. In an F_1 animal responsiveness would be codominant, because any self receptor that mutated to recognize the antigen would not be influenced by the other parental anti-self receptor that could not mutate to recognize the antigen. Furthermore, the model had no problem with the loss in the nonresponder of responsiveness to whole families of closely related but non-cross-reacting antigens, since if the self receptor could not mutate to recognize a given antigen x_1, it is also likely that the set of receptors generated would not see x_2 or x_3.

In order to account for the phenomenon of MHC-restriction, von Boehmer *et al.* updated the model in 1978. This variation proposed two identical anti-self MHC receptors, both of which had to be occupied to stimulate the T cell. In this case only one of the two receptors was postulated to mutate away from self reactivity and give rise to the antigen-specific repertoire. The other receptor did not mutate and thus became the anti-self receptor in what was then a dual-receptor bearing T cell. The explanation of *Ir* gene function remained the same.

The outstanding feature of the Jerne model was its prediction of the results of responder F_1(b.m.) → nonresponder P_1(irr) chimera experiments. When stem cells with the potential to respond to a given antigen mature in the thymus of a nonresponder, only the receptors capable of recognizing the nonresponder MHC-encoded molecules are selected as self receptors. Thus, the antigen-specific repertoire is generated in these chimeras only from receptors that cannot mutate to recognize the antigen. Therefore, the chimera is expected to be a nonresponder even though its antigen-presenting cells are of responder type. Only positive selection models make *Ir* gene function a consequence of MHC restriction. Neither presentation models nor tolerance models have any intrinsic mechanism that would predict this outcome. These other models must simply superimpose restriction rules on top of presentation or tolerance rules in order to delete F_1(b.m.) → nonresponder P_1(irr) clones that recognize the antigen in association with responder Ia molecules, or attribute the F_1(b.m.) → P_1(irr) chimera results to some sort of haplotype-specific suppression.

On the other hand, the Jerne model did not predict the existence of T cell clones in allogeneic strains capable of recognizing antigen in association with nonresponder Ia molecules (see Section VI,B,2,f), unless one allows for degeneracy in the anti-self receptor. Even so, the deletion of such clones in the F_1 cross between the allogeneic strain and the nonresponder, as demonstrated by Ishii *et al.* (1982b), would not be predicted. In the Jerne model, the repertoire derived from the receptors specific for the responder Ia molecules should not be influenced by the introduction in the F_1 of another positive selection step leading to the additional repertoire derived from the receptors specific for the nonresponder Ia molecules. However, there is one

deletion that would occur during this process. That is the loss of alloreactive clones specific for MHC-encoded antigens of the nonresponder. Thus, if the T cells that recognize the antigen in association with nonresponder Ia molecules are part of the repertoire that is alloreactive toward any nonresponder MHC-encoded antigen, then the Jerne model would predict their absence in the F_1. However, the experiments of Dos Reis and Shevach (1983) have demonstrated that a significant number of these T cells only respond to the allogeneic nonresponder Ia molecule in association with the antigen.

b. Single Receptor Models. In order to circumvent this problem, a modified form of a positive selection model has been proposed by R. Schwartz (1982). In this model, the T cell is postulated to have a single receptor specific for both antigen and the Ia molecule. The repertoire is selected in two phases, first by deletion of clones in the bone marrow via a self tolerance mechanism, and second by positive selection in the thymus for clones with modest affinity ($K_D = 10^{-4}$–10^{-7}) for self-MHC-encoded molecules. In the first phase, high affinity clones specific for self MHC-encoded molecules are eliminated. In addition, T cells specific for other self antigens in association with self MHC-encoded molecules are also eliminated, thus allowing for tolerance to create some holes in the repertoire. In the second phase, the T cells with modest affinity, which are normally not activated by interactions with MHC-encoded molecules, are activated to proliferate, and thus positively selected, in the special environment of the thymus. As a consequence of this positive selection, a significant portion of the potential T cell repertoire is lost, i.e., those T cells with low affinity ($K_D < 10^{-4}$) for MHC-encoded molecules. This will place large holes in the T cell repertoire that could serve as a means to explain *Ir* gene function. Finally, the selected T cells that emerge into the periphery are only activated when they encounter foreign antigens in addition to a self Ia molecule. Because the receptor has only a single combining site, steric constraints could force the Ia molecule and antigen to physically interact. This could lead to certain situations (e.g., the cytochrome *c* data discussed in Section VI,B,2,b) in which Ia molecule/antigen interactions determine T cell activation and therefore *Ir* gene control. A similar single receptor model was independently published by Droege (1981).

c. Constraints on Positive Selection Models. Because the discarding in the thymus of very low affinity clones for one Ia molecule should be independent of the same process operating on T cells specific for another Ia molecule, positive selection models predict that there should be instances in a (responder×nonresponder)F_1 in which T cells specific for antigen and nonresponder Ia molecules are positively selected. These clones would not be present in the nonresponder parent. It is possible that the (B6×A)F_1 clone described by Kimoto *et al.* (1981) (see Section VI,B,2,f), which is specific for

(T,G)-A--L in association with the nonresponder Ia molecule of the A strain, is an example of such an F_1 T cell. Another possible example in a cytotoxic T cell system is detailed in Section VIII,C,3 (Billings *et al.*, 1978a,b). However, if positive selection were the dominant mechanism of *Ir* gene control, one would expect to see examples of this type more often. In particular, crosses between two nonresponder strains should occasionally give rise to a responder F_1 strain. Complementation in a positive selection model would produce responders capable of recognizing antigen in association with one of the nonresponder parental Ia molecules. So far, however, all observed *Ir* gene complementation appears to be entirely explained by combinatorial shuffling of Ia molecule subunits to produce unique F_1 restriction elements (see Section V,B,2).

To date, the only experimental evidence to support a positive selection model is the nonresponsiveness of (responder×nonresponder)F_1(b.m.) → nonresponder(irr) chimeras. However, since these animals cannot respond to any antigens in association with the responder Ia molecules, this result does not eliminate an antigen-presentation model for nonresponsiveness. In addition, since the nonresponder animal itself cannot stimulate clones specific for the antigen in association with the nonresponder Ia molecule, the absence of such clones in the chimera could be due to a tolerance mechanism. Thus, although the chimera results can be explained if MHC restriction and *Ir* gene control occurred in a single positive selection step in the thymus, there is no compelling evidence that says that this is the way that *Ir* gene effects arise.

In order to obtain direct evidence for thymic selection as a basis for *Ir* gene effects, Z. Kovac and R. H. Schwartz (unpublished observations) examined the nonresponsiveness of the [B10.A(4R)×B10.PL]F_1 mouse to pigeon cytochrome *c*. Recall from Section V,B,3 that this F_1 is unique among crosses between B10.A(4R) (E^k_β) and E_α-bearing strains. Only B10.PL (E^u_α) failed to complement with E^k_β to produce a responder strain. A biochemical examination of the problem revealed that E^u_α preferentially associated with E^u_β over E^k_β. The result was that [B10.A(4R)×B10.PL]F_1 B cells and macrophages had 1/8 as much E^k_β:E^u_α Ia molecules on their surface, compared to E^k_β:E^k_α Ia molecules on the surface of homozygous B10.A or B10.BR cells. The question then arose as to how this quantitative deficiency in Ia molecule expression was responsible for the nonresponsiveness of the [B10.A(4R)× B10.PL]F_1.

Because [B10.A(2R)×B10.PL]F_1 mice, which possess all the same MHC gene products, were responders to pigeon cytochrome *c*, a tolerance mechanism could not be invoked to explain the nonresponsiveness. Therefore, the defect had to lie either at the level of antigen presentation, at the level of thymic selection or at both levels. In order to examine these potential effects

separately, radiation-induced bone marrow chimeras were set up using B10.A and [B10.A(4R)×B10.PL]F_1 mice. F_1(b.m.) → B10.A(irr) chimeras provided animals in which the developmental environment contained normal expression of E^k_β:E^k_α Ia molecules, but the antigen-presenting cells in the periphery had a quantitative decrease (1/8) in the amount of E^k_β:E^u_α Ia molecules. In contrast, B10.A(b.m.) → F_1(irr) chimeras provided animals in which the developmental environment had the quantitative deficiency in Ia molecule expression, while the antigen-presenting cells had normal amounts of E^k_β:E^k_α on their surface. When these chimeras were immunized with pigeon cytochrome *c*, the B10.A(b.m.) → F_1(irr) chimeras responded and the F_1(b.m.) → B10.A(irr) chimeras did not. This result demonstrated that the primary *Ir* gene defect was at the level of the antigen-presenting cell and not at the level of thymic selection. Surprisingly, a potential 8-fold decrease in thymic Ia molecule expression had almost no effect on the quantitative level of the response to pigeon cytochrome *c*. Compared to B10.A(b.m.) → B10.A(irr) pseudochimeras, the mean proliferative response was only 2-fold lower, and when normalized to the PPD response was not significantly different. Similar results were obtained for the syngeneic MLR to E_β:E_α Ia molecules in these chimeras.

One assumes that the educating cells in the thymus would also express the quantitative Ia molecule defect of the peripheral B cells and macrophages, although no direct evidence for this is available, because no one knows which cell is the educating cell. However, if this is the case, then the positive selection process must have some way of overcoming the quantitative deficiency in Ia molecule expression. One possibility would be if the cytochrome *c*-specific T cell clones had receptors whose affinity for self Ia molecules was on the upper end of the low affinity spectrum (e.g., $K_D = 10^{-7}$). If so, they might be positively selected in the thymus even though the Ia molecule density was 8-fold lower. However, the syngeneic MLR results suggest that this relatively normal selection is occuring for other E_β:E_α-restricted T cells as well. Thus, if quantitative decreases in Ia molecule expression are similar to qualitative (allelic) differences in Ia molecule expression for any given antigen, then the results with pigeon cytochrome *c* suggest that positive selection may not be a dominant force in *Ir* gene control.

4. Suppression

a. Poly(Glu^{60},Ala^{30},Tyr^{10})$_n$ (GAT). In the three forms of repertoire constraints discussed so far, the Ia molecule exerts its influence prior to the introduction of antigen, i.e., during the ontological development of the T cell repertoire. The net result is the absence of T cell clones with receptors

capable of recognizing the foreign antigen in association with nonresponder Ia molecules. In the present section we will consider a completely different mechanism that begins to operate after the introduction of antigen. In this case, T cells do exist with specificity for antigen and nonresponder Ia molecules. However, these cells are actively prevented from functioning by the activation of antigen-specific suppressor T cells. It is not the purpose of this review to outline suppressor T cell circuitry and function. The reader is referred to other reviews for these details (Tada and Okumura, 1979; Germain and Benacerraf, 1980; Tada, 1984; Dorf and Benacerraf, 1984). Here we will only discuss how these cells could relate to *Ir* gene function.

The first *Ir* gene regulated immune response to be shown to involve suppressor T cells was the response to poly(Glu^{60},Ala^{30},Tyr^{10})$_n$ (GAT) in mice (Gershon *et al.*, 1973). An analysis of the genetic control of the antibody response to GAT was initially performed by Martin *et al.* (1971). Strains possessing the *H-2*^{p}, *H-2*^{q}, and *H-2*^{s} haplotypes were found to be nonresponders to GAT and all other strains were found to be responders. The nonresponder strains could produce antibody to GAT if the antigen was electrostatically coupled to a carrier molecule, methylated bovine serum albumin (MBSA), suggesting that the defect was at the T cell activation level (Gershon *et al.*, 1973). Subsequent studies by R. Schwartz and Paul (1976) supported this conclusion by demonstrating that the nonresponder strains were incapable of making a secondary T cell proliferative response *in vitro*. Identical results were also obtained for DTH responses by Miller *et al.* (1977). Thus, in all assay systems, GAT appeared to be similar to other antigens whose responses were under *Ir* gene control, i.e., T cells specific for antigen and nonresponder Ia molecules were not observed.

The first indication that the GAT system might be different came from the studies of Gershon *et al.* (1973). They measured the uptake of ^{125}IUdR by thymocytes following adoptive transfer to syngeneic irradiated recipients and immunization with antigen. In all strains tested immunization with sheep red blood cells produced a significant increase in ^{125}IUdR incorporation over saline controls 2 to 4 days later. In contrast, immunization with poly(Glu^{90},Tyr^{10})$_n$ (GT), to which all inbred strains were nonresponders, did not produce a significant increase in ^{125}IUdR uptake. The results with GAT appeared paradoxical. Immunization of a responder strain (DBA/2, *H-2*^{d}) with GAT did not result in significant ^{125}IUdR uptake, but immunization of a nonresponder strain (DBA/1, *H-2*^{q}) did produce an increased uptake. This suggested that the nonresponder T cell population was recognizing the antigen in some way.

Transfer of these primed spleen cells to a second irradiated syngeneic host and boosting with GAT revealed a large ^{125}IUdR incorporation by responder T cells. In contrast, the uptake by nonresponder T cells boosted with GAT

was actually reduced compared to these same cells boosted with saline. Gershon interpreted these results to mean that both responders and nonresponders recognize GAT, but in different ways. Recognition in the responders was postulated to lead to immunologic memory and an amplified T cell proliferative response. Recognition in nonresponders was postulated to lead to paralysis and elimination of any response.

To demonstrate that this paralysis was indeed an active process, the authors performed an experiment that has now become the classic way of demonstrating suppressor T cells in nonresponder animals. Nonresponder mice were first immunized ip with GAT in saline and then 5 days later challenged with GAT coupled to MBSA. These mice failed to make anti-GAT antibody, in contrast to controls not preimmunized with GAT. Pretreatment of responder mice had no effect. Subsequent studies by Kapp *et al.* (1974a) confirmed these observations and demonstrated that the effect of preimmunization was antigen specific. Thus, GAT appeared to induce a specific T cell response in nonresponder animals that prevented GAT-specific B cell responses, presumably by inhibiting helper T cell function.

The detailed analysis of this system was pursued by Kapp *et al.* (1973a,b) after they developed an *in vitro* primary plaque-forming cell assay for GAT. Mixing GAT primed spleen cells from nonresponder DBA/1 mice with unprimed DBA/1 spleen cells prevented the latter from developing a GAT-specific PFC response when challenged with GAT coupled to MBSA (Kapp *et al.*, 1974b). The cells from primed spleens that mediated this suppression were shown to be radiosensitive (800 R) T cells. Subsequent experiments with sonicates from these spleens demonstrated the presence of a suppressor factor with all of the same properties as the cells (Kapp *et al.*, 1976, 1977). Thus, priming nonresponder mice with GAT appeared to induce antigen-specific suppressor T cells.

The second unusual result found in the GAT system was with (responder $\times$nonresponder)F_1 mice (C. Pierce *et al.*, 1977). Normal spleen cells would develop GAT-specific PFC responses when stimulated with GAT-pulsed nonresponder as well as responder macrophages. In contrast, if the F_1 mice were primed with GAT, then only responder macrophages would present GAT. This result suggested that the F_1 animal possessed T cells capable of recognizing GAT in association with either responder or nonresponder Ia molecules (presumably separate subsets), but that after immunization with GAT, the subset recognizing GAT in association with nonresponder Ia molecules was preferentially suppressed. This result is dramatically different from the studies of the copolymers GL in the guinea pig or GA and (T,G)-A--L in the mouse, where no response to the antigen in association with nonresponder Ia molecules was detected when F_1 T cells were stimulated or primed *in vitro* with antigen-pulsed nonresponder macrophages (see Section III,C).

The demonstration that suppressor T cell induction occurred in the GAT system was shown conclusively by C. Pierce and Kapp (1978b) and Germain and Benacerraf (1978). GAT-primed F_1 spleen cells were shown to suppress the response of normal F_1 spleen cells, but only to GAT-pulsed nonresponder parental macrophages. This suppression was eliminated when the mice were pretreated with cyclophosphamide prior to priming or with anti-*I-J* subregion antisera during the first 4 days postpriming, both procedures known to decrease suppressor T cell activity (Germain and Benacerraf, 1978). Suppression of the response to GAT-pulsed responder macrophages could also be achieved, if F_1 spleen cells were primed with GAT-pulsed nonresponder macrophages instead of soluble antigen (C. Pierce and Kapp, 1978b). In this case, the primed F_1 spleen cells responded to GAT-pulsed nonresponder and F_1 macrophages but not GAT-pulsed responder macrophages. This pattern reverted to the usual suppression of nonresponder macrophage presentation when soluble GAT was administered at the same time as the priming with GAT-pulsed nonresponder macrophages. The same was true for priming with GAT-pulsed F_1 macrophages. In this case, the primed F_1 spleen cells responded to both GAT-pulsed responder and nonresponder macrophages, but if soluble GAT was administered during the priming regimen, then only GAT-pulsed responder macrophages stimulated a PFC response.

These results suggested that the failure of (responder×nonresponder)F_1 spleen cells to respond to GAT-pulsed nonresponder macrophages after priming with soluble GAT was because suppressor T cells prevented the priming of helper T cells specific for GAT associated with nonresponder Ia molecules. These suppressor T cells did not suppress helper T cells specific for GAT associated with responder Ia molecules. The latter clearly could be suppressed if the system was manipulated. This was confirmed by Pierres and Germain (1978) who depleted responder spleen cell populations of macrophages and demonstrated that the cultures now generated suppressor T cells in response to GAT rather than helper T cells. Therefore, the failure to suppress F_1 T cells recognizing GAT in association with responder Ia molecules, at the same time that suppression of F_1 T cells recognizing GAT in association with nonresponder Ia molecules occurs, must result from temporal or quantitative differences in the activation of helper versus suppressor T cells.

To explain these differences Germain and Benacerraf (1978) have put forth the following hypothesis: (1) the helper T cells specific for GAT in association with responder Ia molecules form a quantitatively larger or qualitatively better (e.g., high affinity) subset than the helper T cells specific for GAT in association with nonresponder Ia molecules; (2) suppression is a normal occurrence with antigen priming; soluble antigen causes a rapid induction of suppressor cells, whereas antigen-pulsed macrophages favor stimulation of

helper T cells, possibly because the former recognize free antigen, whereas the latter recognize antigen only in association with Ia molecules; (3) immunization of F_1 mice with soluble GAT rapidly stimulates T helper cells specific for GAT in association with responder Ia molecules, allowing them to escape suppression either by quantitatively increasing their numbers or qualitatively becoming resistant to suppressive signals. In contrast, the smaller number or lower affinity helper T cells specific for GAT in association with nonresponder Ia molecules do not respond fast enough and are suppressed. A quantitative form of this theory, termed the balance of growth model, has been proposed by Grossman (1984).

The important implication of this model for *Ir* gene function is that the genetic control is placed in the macrophage–helper T cell interaction. The induction of suppressor cells is an epiphenomenon, that only comes to regulate the response in an apparent haplotype-specific manner as a secondary consequence of the absence of sufficient haplotype-specific helpers. The model predicts that nonresponder mice should generate less T cell help than responders, a prediction that has been confirmed by pretreating nonresponder mice with cyclophosphamide to eliminate suppressor T cells and detecting only weak T cell helper activity (R. N. Germain, personal communication; Debre' *et al.*, 1976b). It also predicts that the suppressor cells themselves are not directly influenced by the Ia molecule. The only evidence for direct *MHC* gene product involvement in the GAT suppressor response was the presence of *I-J* encoded determinants on suppressor T cell factors (Webb *et al.*, 1983). However, the recent molecular biology findings that *I-J* does not appear to encode a previously undefined structural product of the MHC which is expressed by T cells, makes this evidence moot (Steinmetz *et al.*, 1982; Kobori *et al.*, 1984; Kronenberg and Taniguchi, 1984). In addition, C. Pierce and Kapp (1978b) demonstrated that F_1 spleen cells primed with GAT-pulsed macrophages could suppress the primary GAT response to third party, allogeneic macrophages. These macrophages do not express Ia molecules of the immunizing type and, therefore, could not interact with haplotype-specific suppressor T cells. Thus, the idea that the primary defect is one of too little help for the nonresponder haplotype seems reasonable.

Nonetheless, this hypothesis does not explain all of the data in the GAT system. For example, GAT-pulsed nonresponder macrophages stimulate a primary PFC response from F_1 spleen cells, but these same macrophages do not stimulate a primary PFC response from nonresponder spleen cells (Kapp *et al.*, 1975; C. Pierce and Kapp, 1978b). The nonresponder does contain T cells specific for GAT in association with nonresponder Ia molecules, because, as shown by Araneo and Kapp (1980), if one primes these animals with GAT:MBSA in CFA, the draining lymph node T cells will proliferate in

response to uncomplexed GAT in association with syngeneic nonresponder macrophages. These results, if interpreted from the quantity of help point of view, would mean that the F_1 spleen cells must have a higher frequency of T cells specific for GAT in association with nonresponder Ia molecules than do nonresponder spleen cells. The only way that this might happen is in a positive selection model (see Section VI,C,3) in which the repertoire selected for recognition of responder or unique F_1 Ia molecules would contain clones that were specific for GAT in association with nonresponder Ia molecules.

On the other hand, the difference between the F_1 and the nonresponder strains could be in the quantity of suppressor T cells they possess. Although the Ia molecule in the GAT system does not appear to be involved in suppressor cell activation, the possibility that the Ia molecule influences the suppressor T cell repertoire during development has never been excluded. One hypothesis for how this might occur has been suggested by R. Schwartz (1982). If uncommitted (for help versus suppression), T cells were positively selected via low affinity for self Ia molecules in the thymus, then the way in which these cells first encounter antigen in the periphery might determine which differentiation pathway they follow. Recognition of antigen in association with Ia molecules could lead to differentiation to become a helper cell, whereas recognition of antigen alone could lead to differentiation to become a suppressor cell. Since the receptor and its affinity for activation would be the same during either event, the antigens recognized with and without Ia molecules would necessarily be different. Thus, on any given antigen, determinants recognized by suppressor and helper T cells would not be the same. In this type of model, the Ia molecule could clearly influence the suppressor T cell repertoire and the difference in the quantity of suppressors in the F_1 versus the nonresponder strain could stem from a gene dosage effect (see Section V,B,4).

b. Unique GAT and Lysozyme Determinants Recognized by Suppressor T Cells. Experimental evidence to support the concept of unique suppressor determinants on antigens was first developed in the GAT system (M. Schwartz *et al.*, 1976). Mice possessing the $H\text{-}2^s$ haplotype are nonresponders to both the GAT and GT copolymers but responders to the GA copolymer. The discovery of suppressor cells to GAT and GT in these mice led Waltenbaugh, Dorf, and Benacerraf to speculate that the tyrosine residues might be critical for generation of such T cells. In collaboration with Schwartz, Celsa, and Sela they synthesized an analog of GA in which oligotyrosines were attached to the C-terminus. This new polymer, denoted (G,A)-T, showed a strain distribution of responsiveness similar to that of GAT, i.e., $H\text{-}2^p$, $H\text{-}2^q$, and $H\text{-}2^s$ haplotype-bearing mice were low to nonresponders. Furthermore, (G,A)-T induced suppressor T cells in mice bearing

the $H\text{-}2^s$ haplotype. In particular, preimmunization with (G,A)-T prevented $H\text{-}2^s$-bearing mice from subsequently responding to immunization with GA or GAT:MBSA. Thus, the analog behaved in all respects like the random GAT copolymer. Therefore, it was concluded that oligo-L-tyrosine sequences constituted unique determinants recognized by suppressor T cells from $H\text{-}2^s$-bearing mice and that these determinants were distinct from the determinants composed of L-glutamic acid and L-alanine, which stimulated helper T cells in these strains.

Subsequent to this initial observation, a number of examples of suppressor determinants were found in protein antigens (see Sercarz *et al.*, 1978, for a review). One particularly well studied case is in hen egg white lysozyme (HEL). The response to this antigen is under *Ir* gene control: mice possessing the $H\text{-}2^b$ haplotype are low responders, mice possessing the $H\text{-}2^d$ haplotype are intermediate responders, and mice possessing the $H\text{-}2^{a/k}$ or $H\text{-}2^q$ haplotype are high responders (Hill and Sercarz, 1975). Using HEL coupled to goat or burro red blood cells (RBC) as a carrier molecule, Adorini *et al.* (1979a) were able to demonstrate the presence of HEL-specific suppressor T cells in $H\text{-}2^b$-bearing mice. Immunization with HEL-RBC led to both IgM and IgG PFC specific for HEL. Preimmunization (4 weeks) with high doses of HEL (100 μg) in CFA reduced the subsequent PFC response to HEL-burro RBC by 75–80%, but did not affect the PFC response to burro RBC alone. Preimmunization with just CFA had no effect. Using an *in vitro* culture system for generating a primary PFC response to HEL-sheep RBC, they showed that spleen cells from HEL-CFA-primed mice would suppress the response to HEL-sheep RBC of spleen cells from control mice primed only to CFA. This suppressive activity was eliminated by prior treatment of the cells with an anti-T cell reagent and complement or an anti-$I\text{-}J^b$ reagent and complement. Thus, antigen-specific, $I\text{-}J^+$ suppressor T cells were generated in $H\text{-}2^b$-bearing mice in response to HEL immunization.

The ability to chemically and enzymatically dissect protein antigens was then used to localize the determinant on the lysozyme molecule responsible for inducing these suppressor T cells (Adorini *et al.*, 1978b). Mild acid hydrolysis of HEL produced a fragment called N-C which contains residues 1–17 from the amino terminal end of the molecule covalently coupled by a disulfide bond (Cys-6 to Cys-127) to residues 120–129 from the C-terminal end. This fragment was fully capable of inducing suppressor T cells in B10 mice, suggesting that it contained the suppressor determinant. Further localization of the determinant to the amino-terminus around residue 3 was achieved by studying lysozymes from closely related species of birds. Lysozymes from Bob White quail (BEL), Gamble quail (LEL), Valley quail (VEL), and Guinea-Hen (NEL) were all nonimmunogenic in $H\text{-}2^b$-bearing mice, whereas lysozymes from Japanese quail (JEL) and ringed neck pheas-

ant (REL) were immunogenic. A comparison of the sequences of these proteins revealed that the immunogenic ones possessed a tyrosine at position 3 whereas the nonimmunogenic ones possessed a phenylalanine at this position.

In the process of localizing the suppressor determinant, Sercarz's group discovered that another fragment of lysozyme, LII (a reduced and carboxymethylated cyanogen bromide cleavage fragment comprising residues 13 to 105), was immunogenic in $H\text{-}2^b$-bearing mice (Yowell *et al.*, 1979). Priming B10 mice with LII yielded T cells capable of proliferating in response to LII or HEL. In contrast, immunization with HEL resulted in no detectable proliferative response to either the fragment or the whole molecule. However, when HEL primed T cells were mixed with LII primed T cells at a ratio of 1 to 4, the response of the latter cells to HEL was suppressed, but their response to LII was not. This result was interpreted to mean that the HEL primed population contained suppressor T cells specific for the N-terminal determinant and that these cells could suppress the proliferation of T cells specific for another determinant(s) on the LII fragment of the molecule. The suppressor cells could only function when the suppressor determinant was present, i.e., with HEL and not with LII. Note that this is a different result from what was observed in the GAT system, where pretreatment with (G,A)-T suppressed the response to GA. In addition, recent experiments in the lysozyme system (E. E. Sercarz, personal communication) have shown that introduction of the N-C peptide into the cultures along with the LII peptide was *not* sufficient to suppress the response to the LII determinant. This observation suggested that the suppression was mediated by an antigen bridge connecting the two T cells (helper and suppressor) via their recognition of different determinants on the molecule.

This conclusion was supported by a temporal separation of the emergence of suppression and help in nonresponder mice. Araneo *et al.* (1979) immunized C57BL/6 mice with HEL in CFA in the hind foot pads and examined the draining popliteal and inguinal lymph node cells at various times after immunization. In contrast to what had been observed for splenic T cells, they found that at day 7–10 a significant amount of helper activity could be detected in the lymph nodes if the animals were immunized with 50 μg of antigen. However, at day 14 there was a sudden, 70% decrease in helper activity that persisted out to day 21. This dramatic decrease was not observed in responder strains nor in C57BL/6 mice immunized with REL, a lysozyme to which B6 mice respond. Mixing day 21 lymph node T cells with day 10 T cells decreased the helper activity of the latter to that of the former. This suppression was shown to be caused by $I\text{-}J^-$ T cells in the day 21 population interacting with $I\text{-}J^+$ T cells in the day 10 population in a suppressor circuit similar to those described in detail for other antigens (see

Germain and Benacerraf, 1981; D. Green *et al.*, 1983; or Tada, 1984, for a review of this topic).

Overall, the results suggested that suppressor T cells arise early in the spleen in response to the N-terminal determinant on HEL and prevent any helper T cells from emerging in this organ. In contrast, draining lymph node populations do not initially appear to contain suppressor precursors. Therefore, they initiate a helper response to the LII portion of the antigen. However, by day 14, cells in the suppressor series enter into the lymph nodes and neutralize these helper T cells by an antigen-bridging mechanism. As a consequence, the animal is a nonresponder to HEL (although it is capable of responding to the LII fragment).

This explanation of the data proposes that nonresponsiveness is a consequence of the existence of suppressor T cells in the nonresponder strain capable of recognizing the suppressor determinant. Does this mean that the responder strain is capable of recognizing HEL because it lacks these suppressor T cells? Not necessarily. The presence of suppressor T cells in one *H-2* congenic strain but not another does not prove that the difference between them is the influence of their Ia molecules on the suppressor T cell repertoire. It could instead reflect the influence of the Ia molecule on T cell help for specific determinants. For example, in the case of HEL, the suppressor T cells are capable of nullifying helper T cells specific for determinants on the LII fragment, but they would not be capable of affecting helper T cells specific for the N-terminal region of the molecule, containing the suppressor determinant, at least not in an antigen-bridging model. Interestingly, the B10.A responder strain, when immunized with the N-C fragment of HEL containing the suppressor determinant, is primed for as much helper activity as when it is immunized with the whole molecule (Adorini *et al.*, 1979b). Thus, the *Ir* gene control could be primarily exerted at the level of activation of helper T cells specific for the N-terminal determinants: responders recognize these determinants and helper cells dominate, whereas nonresponders fail to recognize these determinants and the suppressor cells specific for this region emerge as the dominant force. In this model the suppressor T cells potentially are present in both strains and their emergence in the nonresponder is secondary to the absence of a helper T cell with a particular specificity.

c. Insulin: The Absence of Help versus the Presence of Suppression. The clearest example of this model comes from the recent work of Jensen *et al.* (1984) on the immune response to beef and pork insulin in mice of the $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes. B10 mice are responders to beef insulin and nonresponders to pork insulin (Keck, 1975). However, pork insulin can prime B10 mice to provide T cell help for B cells in a secondary antibody response to beef insulin *in vivo* or *in vitro* (Bucy and Kapp, 1981, 1983). An analysis of

this phenomenon revealed that immunization with pork insulin had two effects (Jensen *et al.*, 1984). One was that helper T cells specific for a determinant on the B chain were primed. The second was that suppressor T cells specific for a determinant in the alpha loop of the A chain were primed. The latter inhibited the former from functioning. However, if beef insulin was used in the secondary response, it worked because this molecule contained amino acid differences in the alpha loop that prevented the suppressor T cells from recognizing it. Since beef and pork insulins have identical B chains, the helper T cells primed to the B chain determinant could now function to produce an antibody response.

In contrast, when B10 mice were primed with beef insulin, no suppressor T cells for pork or beef insulin could be detected. Instead, Jensen *et al.* (1984) and others (Rosenwasser *et al.*, 1979; Reske-Kunz and Rüde, 1982) found helper T cells specific for the alpha loop. This was the dominant helper population, although other laboratories have reported clones of T cells specific for determinants involving the B chain (Hochman and Huber, 1983). Thus, in the presence of a large response to the alpha loop determinant of beef insulin, the suppressor cells were missing. However, immunization of mice bearing the $H\text{-}2^k$ haplotype showed that it was possible to generate suppressor T cells specific for beef insulin (Jensen *et al.*, 1984). These suppressor cells functioned in the same way as the pork insulin-specific suppressors in B10 mice in that they suppressed the response of helper T cells specific for determinants on the B chain.

Thus, the question arises as to why there were no suppressor cells with specificity for the alpha loop in B10 animals immunized with beef insulin? Two possibilities should be considered. One is that suppressor T cells are specific for the alpha loop determinant in association with $H\text{-}2^k$-encoded Ia molecules and therefore could not be primed in B10 mice. The other is that the helper response to the alpha loop determinant in association with $H\text{-}2^b$-encoded Ia molecules overwhelmed the suppression, because the suppressor cells could not influence this population. This is possibly because the two populations were competing for the same or an overlapping determinant and thus they could not form an antigen bridge. In mice of the $H\text{-}2^k$ haplotype other *Ir* gene mechanisms (deletional or presentational) would preclude the stimulation of helper T cells specific for the alpha loop of beef insulin in association with Ia^k molecules and therefore allow the suppressor T cells to emerge.

To distinguish between these two possibilities, one has to look at the response of F_1 animals. If suppressor cell generation depends on *H-2* haplotype, then the F_1 should generate suppressor T cells that eliminate the response to beef insulin. In contrast, if the presence or absence of helper T cells specific for the alpha loop is the critical variable, then the F_1 animal

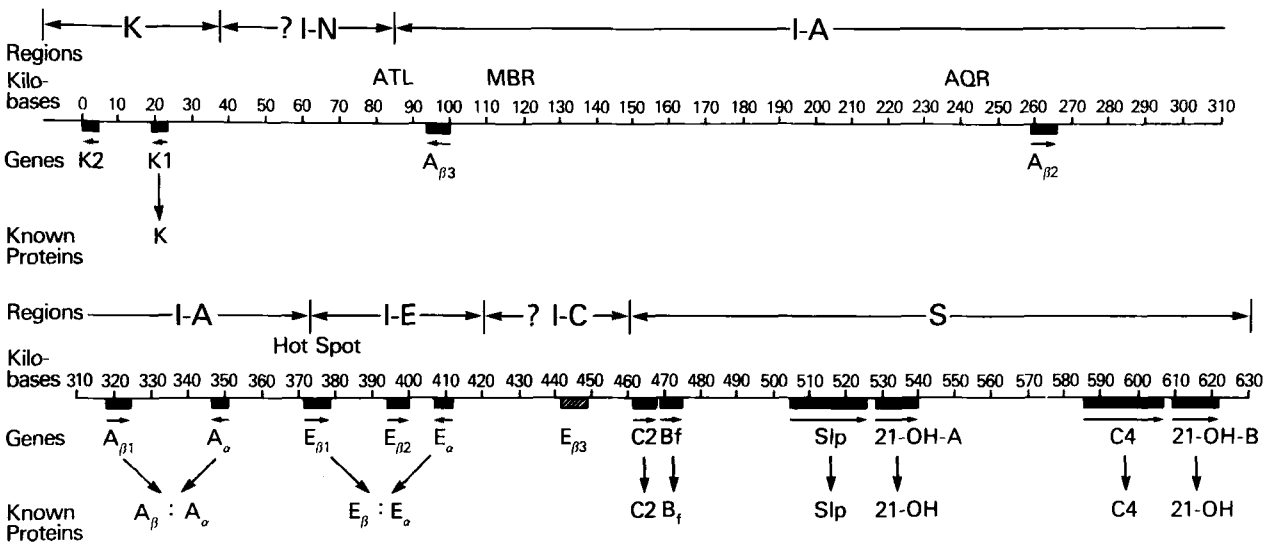
should be a responder. The latter result has been observed by several laboratories. Therefore, the concept of helper cells "protected" from suppression by virtue of their recognition of the same determinant appears to be the best explanation for the experimental results. However, the possibility that the $H\text{-}2^k$-restricted suppressors did eliminate the $H\text{-}2^b$-restricted helpers, but were unable to inhibit the unique F_1-restricted helpers because they recognize a different determinant (Reske-Kunz and Rüde, 1982), has not been ruled out.

d. Poly(Glu^{50},Tyr^{50})$_n$ (GT) and the I-C Subregion. The distinction between the absence of help and the presence of suppression as the primary explanation of *Ir* gene function has always been a difficult one to make. One system that holds great promise for dissecting this problem is the immune response to the random copolymer poly(Glu^{50},Tyr^{50})*n* (GT) studied by Debre' *et al.*, (1975a,b). Although some random-bred swiss mice respond to GT, none of the inbred strains of mice is a responder to this antigen at the helper T cell level, with the exception of the bm12 mutant discussed earlier (Section V,D,1). However, all the strains produced an IgG PFC response in the spleen upon immunization with GT coupled to MBSA. The question of whether suppressor T cells were responsible for the nonresponsiveness to GT was investigated by preimmunizing with GT 3 days before challenging with GT:MBSA. Interestingly, mice bearing the $H\text{-}2^d$, $H\text{-}2^f$, or $H\text{-}2^s$ haplotypes were suppressed by prior GT administration, whereas mice bearing the $H\text{-}2^a$, $H\text{-}2^b$, or $H\text{-}2^q$ haplotypes were not suppressed. In those strains mainfesting suppression, it could be transferred to normal syngeneic recipients by T cells. These results demonstrated that only some forms of nonresponsiveness involved the participation of suppressor T cells. In those strains where no suppression was detected, the underlying mechanism of nonresponsiveness must be the absence of help, presumably as a consequence of one of the 4 mechanisms outlined earlier. Thus, clearly, suppression cannot be a universal explanation of nonresponsiveness.

In those strains in which suppression was found, genetic dominance of this trait turned out to be relative. Crosses between suppressible and nonsuppressible strains produced F_1 hybrids that were suppressible with prior GT immunization. This suggested that the generation of suppressor T cells was an active process that required the presence of MHC-encoded molecules of a particular type. In contrast, as shown in the other systems, i.e., GAT, HEL, and insulin, suppression was recessive to responsiveness, i.e. (swiss responder×nonresponder)F_1s did not generate detectable numbers of the specific suppressor T cells. This result suggested that GT-specific suppression could only be detected in the absence of GT-specific help. Nonetheless, the existence of MHC-congenic mice that could not generate helper or suppressor T cells in response to GT provided the first good evidence that *Ir* genes directly influenced the generation of suppressor T cells.

The genetic control of the generation of GT specific suppressors was surprisingly complex, involving two *I* region genes, one mapping to the *I-A* subregion, the other to the *I-C* subregion (or possibly the S or G regions) of the MHC (Debre' *et al.*, 1976a). The dual *I* region gene control was demonstrated by complementation experiments similar to those described for the *Ir* genes for GLPhe (see Section V,B,1). Crossing the two nonsuppressor-generating strains B10 and B10.A produced an F_1 hybrid that was capable of generating suppressors. That this represented complementation between two separate genes was shown by the demonstration of GT suppressor induction in the B10.A(5R) strain, which bears a recombinant chromosome derived from the two nonsuppressible B10 and B10.A strains. Certain pairs of alleles in *I-A* and *I-C* would complement, others would not (see Dorf, 1978, for a review). The complexity of this complementation can be seen in a comparison of the B10.BR ($H\text{-}2^k$), B10.D2 ($H\text{-}2^d$), and B10.A ($H\text{-}2^{k/d}$) strains. Thus, the *k* allele in *I-A* functioned with the *k* allele in *I-C* (B10.BR) to generate suppressor T cells, but the $I\text{-}A^k$ allele would not function with a *d* allele in *I-C* (B10.A), even though the latter functioned perfectly well with the *d* allele in *I-A* (B10.D2). This selective pairing of alleles was called coupled complementation by Dorf (1978). Its hallmark is the identification of recombinant strains that fail to manifest suppression, e.g., B10.A; yet they are derived from a recombination event involving chromosomes from two strains, both of which are capable of generating suppressor T cells.

The molecular basis for the coupled complementation is unknown. The fact that the *I-C* allelic products of the $H\text{-}2^d$ and $H\text{-}2^k$ suppressor haplotypes are functionally distinct contrasts this complementation with the *Ir* gene complementation in the GLPhe system, where *I-E* encoded α chains from a variety of haploptypes were found to be functionally equivalent (see Section V,B,1). The significance of this can be seen in the current DNA map of the murine *I* region shown in Fig. 4. Functional gene products are known for the A_β and A_α genes (A_β:A_α Ia molecule) and the $E_{\beta 1}$ and E_α genes (E_β:E_α Ia molecule). Because of a DNA "hot-spot," most of the known genetic recombination events occurred in the first intron of the $E_{\beta 1}$ gene between the first and second exons. As a consequence, the *I-A* subregion, defined by these recombination events, encodes not only the A_β:A_α Ia molecule but also the polymorphic, external domain of the $E_{\beta 1}$ protein ($E_{\beta 1-1}$). The second domain of $E_{\beta 1}$ ($E_{\beta 1-2}$) is not very polymorphic (Saito *et al.*, 1983; Mengle-Gaw and McDevitt, 1983; Widera and Flavell, 1984) and neither is the entire E_α protein (McNicholas *et al.*, 1982b; Mathis *et al.*, 1983). Thus, in the gene complementation in the immune response to GLPhe, recombinant strains bearing the $E_{\beta 1-1}$ exon of one allelic form (*b*) can complement with a variety of different strains bearing closely related structural genes for $E_{\beta 1-2}$ and E_α, whose proteins function equivalently to create the E^b_β:E_α Ia molecule required for the GLPhe response.


Regions
K
? I-N
I-A
Kilo-
bases
ATL
MBR
AQR
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310
Genes
K2
K1
$A_{\beta 3}$
$A_{\beta 2}$
Known
Proteins
K
Regions
I-A
I-E
? I-C
S
Hot Spot
Kilo-
bases
310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630
Genes
$A_{\beta 1}$
A_{α}
$E_{\beta 1}$
$E_{\beta 2}$
E_{α}
$E_{\beta 3}$
C2
Bf
Slp
21-OH-A
C4
21-OH-B
Known
Proteins
$A_{\beta} : A_{\alpha}$
$E_{\beta} : E_{\alpha}$
C2
B_f
Slp
21-OH
C4
21-OH

In contrast, a functional equivalence is not found for the products of genes in this MHC region that are involved in the generation of GT suppressors. Equivalence is also not found for a number of other functional responses controlled by genes in this part of the MHC, e.g., an *Ir* gene regulating the antibody response to the copolymer poly(Glu^{80},Phe^{20})$_n$ (GPhe) (Merryman *et al.*, 1978), an *Ir* gene regulating the antibody and T cell proliferative responses to myoglobin (Berzofsky *et al.*, 1979), and a gene(s) influencing the generation of suppressor T cells in the mixed lymphocyte response (Rich and Rich, 1976). In each case, the B10.A(*k*/*d*) and B10.BR(*k*) strains showed a difference in their biological response, whereas the B10.A(*k*/*d*) and B10.D2(*d*) strains behaved similarly. In addition, two laboratories have claimed to raise a B10.A(2R) anti-B10.A(4R) antiserum against a gene product from this subregion (David and Shreffler, 1974; Sandrin and McKenzie, 1981) and such antisera have been reported to inhibit the *I-C* encoded suppressor factor generated in an allogeneic MLR (Rich *et al.*, 1979) and the weak MLR stimulated by a T cell lymphocyte-activating determinant encoded in *I-C* (Okuda and David, 1978). On the other hand, biochemical studies of the E_β:E_α Ia molecules, using two-dimensional gel electrophoresis, demonstrated that

FIG. 4. Molecular organization of the *I* region of the murine MHC. A model of the segment of mouse chromosome 17 containing the *I* region of the MHC is depicted with the centromere (not shown) to the left of the K2 gene. Each gene is shown as a single solid rectangle (although each is composed of several exons), and the direction of transcription is indicated by an arrow below the box. The one exception is the $E_{\beta 3}$ gene which is presented as a hatched rectangle in order to indicate that its precise location in the genome has not yet been determined. The hot spot of genetic recombination that occurs in the intron between the first ($E_{\beta 1-1}$) and second ($E_{\beta 1-2}$) exons of the $E_{\beta 1}$ gene defines the recombination point between the *I-A* and *I-E* subregions. The B10.A(3R), B10.A(4R), B10.A(5R), B10.GD, B10.S(8R), B10.S(9R), B10.HTT, B10.RSB-1, and TBR-2 recombinants all occurred in this area (Hood *et al.*, 1983). The A.TL, MBR, and AQR recombinants, whose cross-overs occurred in the area of the genome just telomeric to the K region, are shown above the approximate area of the DNA where the recombination event took place (Steinmetz, 1985). The *I-N* and *I-C* subregions have been defined on the basis of functional studies (Okuda and David, 1978; Hayes and Bach, 1980; Rich *et al.*, 1979; Dorf, 1981; Merryman *et al.*, 1978) and may or may not correspond to separate *I* region genes and products (Monaco and McDevitt, 1982; Klein *et al.*, 1983). $A_{\beta 1}$, A_α, $E_{\beta 1}$, and E_α are known to encode the functional Ia molecules A_β:A_α and E_β:E_α. $A_{\beta 3}$ is a pseudogene in the $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes that shows strong sequence homology to the human SB locus (Wake *et al.*, 1985). $A_{\beta 2}$ and $E_{\beta 2}$ are transcribed, but no protein products or functions have yet been ascribed (Devlin *et al.*, 1984). Whether $E_{\beta 3}$ is transcribed has not yet been determined; however this gene offers the best potential candidate for explaining the immunological phenomena whose control has been mapped to the *I-C* subregion. The *K* region encodes the MHC class I molecule, K, and the *S* region contains genes that encode the complement components, C2, factor B (B_f) and C4, and the enzyme 21-hydroxylase (21-OH), involved in steroid biosynthesis (White *et al.*, 1984). The C4 and 21-OH genes appear to have duplicated, and the Slp (sex-limited protein) gene product appears to be a "nonfunctional" version of the C4 protein, i.e., it has no C4-like activity.

the *k* and *a* allelic forms of the E_β and E_α proteins were identical, whereas the *d* allelic form was different (Jones *et al.*, 1978). This has been partially confirmed at the DNA level where sequencing of cDNA and/or genomic clones encoding the $E_{\beta 1}$ protein of the B10.A(*k*/*d*) and BALB/c(*d*), revealed one amino acid difference in the second domain, $E_{\beta 1-2}$, while sequencing of the clones encoding the E_α protein of the B10.A(*k*/*d*) and BALB/c(*d*), revealed three amino acid differences, two in the second domain of E_α and one in the transmembrane segment (McNicholas *et al.*, 1982b; Mathis *et al.*, 1983). Because of these structural studies, it was concluded that the E_β:E_α Ia molecule of B10.A was the same as that of B10.BR and different from B10.D2. Therefore, the functional and serologic studies suggested that another genetic region must exist 3′ of *I-E*. Hence the creation of the *I-C* subregion.

At present (see Fig. 4), one gene, $E_{\beta 3}$, encoding a class II β-like protein, has been located downstream (3′) of E_α (Devlin *et al.*, 1984). However, no protein has yet been detected as a product of this gene. Thus, we are currently left with an incomplete picture as to what constitutes *I-C*. The solution will undoubtedly lie either in the complexity of the biology or the subtlety of the DNA differences. For example, the protein sequence of E^a_α might turn out to be different from E^k_α, depending on where the recombination event occurred in the B10.A mouse and whether genetic drift has taken place. On the other hand, the $E_{\beta 3}$ gene, whose expression and function remain unknown, might play an important role in the generation of suppressor T cells. If the product of this gene is expressed only in suppressor T cells, then structural differences in allelic products (B10.A vs. B10.BR) might have been missed. One can rightfully argue now that the use of subregion designations is no longer appropriate and that we should await the complete structural analysis of the genes in this genetic region before attributing a given function to a specific gene product. Nonetheless, a genetic difference must exist somewhere in the genome to explain the biological data!

The cellular basis of the complementation for GT suppression in the (B10×B10.A) and (B6×A/J)F_1s was explored by Germain *et al.* (1980). An analysis of the generation of suppression in a variety of antigen systems has demonstrated that several cell types participate in a suppressor pathway to produce the ultimate suppressor effector T cell (see Germain and Benacerraf, 1981; Green *et al.*, 1983; Tada, 1984). Each cell interaction in the pathway can be supplanted by a factor obtained from the initiating T cell by sonication, which will act in conjunction with the antigen to stimulate the next target cell in the pathway. In the GT system it was discovered that A/J mice fail to produce suppressor cells when stimulated with GT, because they fail to produce the first T cell in the pathway (Ts_1) capable of generating a suppressor factor (TsF_1). This is not the problem in B6 mice, because they

can produce a TsF_1 that will suppress the GT-MBSA response of other strains, including A/J. The problem in B6 is the second cell in the pathway (Ts_2). B6 cells are not affected by TsF_1 either from their own Ts_1 cells or from Ts_1 cells of the $(B6\times A)F_1$ suppressor-generating strain. The defect in the B6 Ts_2 cells was shown to be exquisitely antigen specific in that a GAT-specific TsF_1 would induce GAT-specific Ts_2 cells, but not GT-specific Ts_2 cells. In contrast, the A/J strain does respond to GT-specific TsF_1 from both donors, leading to suppressor cell generation. This indicates that the Ts_2 cell in the A/J is functionally competent. Overall the data suggested that the basis for the *I* region gene complementation in the $(B6\times A)F_1$ was at the cellular level. The B6 haplotype allows the generation of Ts_1, which then can interact with Ts_2 cells generated as a consequence of the A haplotype. However, the exact relationship between each suppressor T cell and the two *Ir* genes is not clear. One gene might be required to generate each suppressor cell as suggested by the complementation. On the other hand, it is possible that both genes will be required in each parental strain to generate the missing suppressor cell. These two models can be distinguished in chimeric animals of the type B6(b.m.) + A/J(b.m.) $\rightarrow$ $(B6\times A)F_1$(irr), which should be capable of generating GT-specific suppressor T cells if the first model is correct, but would not be capable of generating such suppressors if the second model is correct.

e. Lactate Dehydrogenase B (LDH_B) and the I-B Subregion. The GT system clearly demonstrates that the generation of at least some suppressor T cells is an active process that requires certain *I* region-encoded products for the completion of the pathway. However, the direct involvement of these *Ir* gene products (?Ia molecules) in cell interactions, and which cell types express them have not yet been elucidated. Recently, Baxevanis *et al.* (1981, 1982) have been able to accomplish this in another suppressor pathway specific for the antigen lactate dehydrogenase B (LDH_B). In examining the *I-B* subregion, which was only defined based on *Ir* genes (i.e., functionally) and not on the identification of any structural gene product (i.e., Ia molecule), they discovered that a response to LDH_B could be unmasked in certain nonresponder strains either by pretreating primed cells with anti-Lyt 2 and complement or by introducing monoclonal antibodies directed against *I-E* subregion-encoded Ia molecules during an *in vitro* T cell proliferation assay (Baxevanis *et al.*, 1981). For example, B10.A(2R) mice are nonresponders to LDH_B, whereas B10, B10.A(4R), and B10.A(5R) mice are responders. It is this pattern of responsiveness that defines the *I-B* subregion. However, addition of Ia.m7 to cultures of primed B10.A(2R) lymph node T cells allowed this population to respond to LDH_B. This anti-E_α monoclonal had no effect on the B10.A(4R) T cell proliferative response to LDH_B, suggesting that the responding T cells were specific for the antigen in asso-

ciation with the A_β:A_α Ia molecule. This was, in fact, demonstrated by inhibiting the proliferative response of the B10.A(4R) T cells with Ia.m5 or Ia.m2, two monoclonals specific for the A^k_β:A^k_α Ia molecule. If these monoclonals were included along with Ia.m7 in the B10.A(2R) cultures, they also inhibited the unmasked B10.A(2R) response. These results were interpreted to mean that both the B10.A(4R) and the B10.A(2R) respond to LDH_B in association with A^k_β:A^k_α Ia molecules, but that the response of the B10.A(2R) T cells is normally suppressed by another set of T cells specific for LDH_B in association with E^k_β: E^k_α Ia molecules. The B10.A(4R) does not express E^k_β:E^k_α Ia molecules and therefore is a responder. If one further postulates that, in the B10.A(5R) strain, suppressor cells are not generated to LDH_B in association with E^b_β:E^k_α Ia molecules or that such suppressors cannot inhibit proliferative T cells restricted to recognizing LDH_B in association with A^b_β:A^b_α Ia molecules, then this would explain why B10.A(5R) mice are responders to LDH_B and obviate the need for the *I-B* subregion. Similar results were obtained with the IgG_{2a} myeloma protein, UPC 10; the response to this antigen was also thought to be controlled by an *Ir* gene mapping in the *I-B* subregion. The conclusion from these experiments, that the *I-B* subregion does not exist as a structural entity, has been confirmed at the DNA level (Fig. 4).[4]

The negative interaction postulated to exist between the A^k_β:A^k_α-restricted proliferating T cell and the E^k_β:E^k_α-restricted suppressor T cell was further pursued in C3H mice ($I\text{-}A^k$, $I\text{-}E^k$) immunized with LDH_B (Baxevanis *et al.*, 1982). Isolation of the Lyt 1^+2^- subset of T cells by an antibody-coated, plate-binding method, either before or after priming with LDH_B, revealed a population of T cells in this "nonresponder" strain capable of proliferating to LDH_B when presented on glass-adherent peritoneal washout cells. This presentation was inhibited by Ia.m5, the monoclonal antibody directed

[4] Although the DNA studies are unequivocal in their failure to find an *I-B* subregion, a full explanation of the immunological results has not yet been given. For example, in the response to IgG_{2a} one would expect the [B10.A×B10.A(4R)]F_1 to be a nonresponder since the suppressor T cells restricted to E^k_β:E^a_α should act on the helpers restricted to A^k_β:A^k_α of both parents. Although this type of result is what was observed in the response to TNP-mouse serum albumin (see Section VI,C,2,f), it was not observed for the response to MOPC 173, the IgG_{2a} molecule studied by Rose Lieberman (unpublished observations). Perhaps a gene dosage effect accounts for this result? In addition, the response pattern for the antigens staphylococcal nuclease (Lozner *et al.*, 1974) and oxazolone (Fachet and Ando, 1977), are the reciprocal of that for LDH_B and the IgG_{2a} myelomas, i.e., B10.A is a responder and B10, B10.A(4R), and B10.A(5R) are nonresponders. This pattern also gives the illusion of an *I-B* subregion, but no E_β:E_α restricted suppressor T cell scenario can explain the results. Perhaps the function of one of the additional β genes (see Fig. 4, $A_{\beta 2}$ or $E_{\beta 2}$) will eventually provide the answer. Alternatively, maybe pairing of A^k_β and E^k_α chains or E^k_β and A^k_α chains will explain the results (R. N. Germain, personal communication).

against the A^k_β:A^k_α Ia molecule. The proliferative response of the isolated Lyt 1^+2^- T cells was completely inhibited by the addition of a second subset of T cells possessing the Lyt 1^+2^+ phenotype. This inhibition was blocked by the addition of Ia.m7 to the cultures, again demonstrating the requirement for E^k_β:E^k_α Ia molecule recognition by the suppressor T cell.

In order to study the genetic control of the interaction between these two T cell subpopulations, Baxevanis *et al.* (1983) developed an elaborate *in vitro* protocol. First B10.S Lyt 1^+2^- T cells were depleted of alloreactive cells specific for B10.A(2R) MHC-encoded antigens by exposure of the responding cells to bromodeoxyuridine and light. The remaining T cells were then primed to LDH_B on allogeneic B10.A(2R) antigen-presenting cells (APCs). When these cells were rechallenged *in vitro* with LDH_B and B10.A(2R) APCs, a proliferative response was observed, whereas rechallenge with syngeneic B10.S APCs did not stimulate a response. This demonstrated that the B10.S Lyt 1^+2^- T cells were specific for LDH_B in association with B10.A(2R) Ia molecules. If prior to rechallenge *in vitro*, the Lyt 1^+2^- T cells were mixed with LDH_B primed Lyt 1^+2^+ B10.A(2R) suppressor T cells for 6 hours at 37°C and then the suppressor cells removed by treatment with anti-Lyt 2 and complement, the remaining Lyt 1^+2^- B10.S T cells did not proliferate to LDH_B in association with B10.A(2R) APCs. In the converse experiment, i.e., B10.A(2R) Lyt 1^+2^- T cells primed to LDH_B on B10.S APCs, the B10.A(2R) Lyt 1^+2^+ suppressor T cells had no effect. These results demonstrated that the receptor specificity of the proliferating T cell, and not its expressed MHC phenotype, is what was critical for observing the suppression.

The mechanism by which the restricted interaction occurred was elucidated by deriving a suppressor T cell hybridoma specific for LDH_B and isolating from it an antigen-specific, *I-A*-restricted suppressor factor (Ikezawa *et al.*, 1983). This factor suppressed an LDH_B-primed, Lyt 1^+2^- proliferative T cell population specific for LDH_B in association with the A^k_β:A^k_α Ia molecule. The interaction between the suppressor factor and the proliferative T cell occurred through an antigen bridge and required the recognition of an "Ia-like" determinant on the factor. This determinant did not react with conventional anti-Ia monoclonal antibodies specific for B cell-derived A^k_β:A^k_α Ia molecules, but it did react with T cell-specific monoclonal "anti-A^k" antibodies. The nature of the molecule bearing this "Ia-like" determinant is not clear. Most molecular biologists have not been able to detect mRNA encoding conventional class II molecules in suppressor T cell hybridomas (Kronenberg and Taniguchi, 1984; Hodes *et al.*, 1984; R. I. Lechler and R. N. Germain, unpublished observations) although exceptions have been reported (Koch *et al.*, 1983; Singh *et al.*, 1984). An alternative possibility that has been suggested (Schrader, 1979; Sim *et al.*, 1984; Hodes *et al.*, 1984) is that the determinant is a T cell "internal image" of a B cell Ia

determinant present on the antigen-specific receptor of the suppressor T cell. But no matter what the explanation, the experiments suggest that the proliferating T cell recognizes on the suppressor factor a configuration similar to that which it first encountered on the antigen-presenting cell. This interaction inhibits the T cell from responding in a manner that appears to be irreversible, at least within the time frame of the experiments.

The important new finding in this work is that a clearly defined suppressor T cell was activated by recognition of antigen in association with an Ia molecule. In fact, normal T cell clones with this phenotype have also been isolated (Asano and Hodes, 1983). This allows one to argue that *Ir* genes can directly influence the suppressor T cell repertoire through any of the five other mechanisms discussed earlier: presentation, tolerance, positive selection, repertoire limitations, or limitations in antigen processing. Thus, it now becomes possible to imagine that the difference between a nonresponder and a responder to a particular antigen is solely the result of a difference in the quantity or quality of the suppressor T cell repertoire of the strain rather than a difference in the helper population, i.e., active suppression could be the primary cause of nonresponsiveness and not an epiphenomenon.

D. Summary

Table III provides a summary of the different mechanisms detailed in the previous sections to account for the antigen-specific effects of Ia molecules on the immune response. Although strong diatribes have appeared in the literature (Janeway, 1983; Klein, 1984) extolling the virtues of one mechanism over another, my own opinion is that most of these mechanisms probably play some role in determining *Ir* gene-controlled nonresponsiveness. In any one case a particular mechanism may appear to dominate; but at this point in time I see no compelling evidence to argue that one mechanism is always dominant. It is possible that the distinctions that I have made with regard to different mechanisms will begin to blur upon closer scrutiny. For example, some forms of tolerance may turn out to be T cell suppression mechanisms for self antigens, or presentation of self antigens in the thymus may turn out to be a key element in positive selection. Nonetheless, what matters in these models is that they serve as working hypotheses to help us design experiments to explore the molecular basis of the cellular interactions required for T cell development and activation. The antigen-specific effects will certainly be clarified when we understand the way in which the T cell receptor, the Ia molecule, and the antigen interact during selection and stimulation.

TABLE III
POSTULATED MECHANISMS FOR *Ir* GENE-CONTROLLED IMMUNOLOGICAL NONRESPONSIVENESS

1. Limitations in the germline-encoded T cell *V* region repertoire
2. Elimination or inactivation by a tolerance mechanism of T cell clones specific for self MHC-encoded molecules and other self antigens, either alone or in association
3. Failure to positively select T cell clones during thymic education
4. Inability of the MHC-encoded molecule and the antigen to physically interact in a favorable way during antigen presentation
5. Limitations in the nature or number of antigen fragments produced during protein degradation (processing)
6. Preferential generation of suppressor T cells specific for antigen in association with Ia molecules

VII. *Ir* Gene Control of T Cell–T Cell Interactions

A. (Tyr,Glu)-DL-Ala--Lys[(T,G)-A--L] AND HELPER FACTORS

In most models of *Ir* gene function, the Ia molecules, which are the *Ir* gene products, are recognized by T cells on the surface of other cell types such as antigen-presenting cells or B lymphocytes. Thus, the T cell has an anti-Ia receptor and not an Ia molecule on its surface. However, a number of pieces of data in the literature have suggested that this might not always be the case. Under some circumstances it appears as if T cell–T cell interactions are restricted by gene products of the MHC (see Germain and Benacerraf, 1981; D. Green *et al.*, 1983; Tada, 1984, for reviews). This is a highly controversial area since the major genetic locus thought to be involved in such interactions, *I-J*, has recently been shown not to be a separate locus in the MHC encoding a T cell product (Kobori *et al.*, 1984; Hayes *et al.*, 1984; Kronenberg and Taniguchi, 1984). Nonetheless, MHC-encoded molecules clearly influence these interactions in some indirect way. If so, one might expect to observe antigen-specific defects in the pathways, which could be considered as *Ir* gene control. Therefore, this section will review the experiments in this area in order to illustrate some possible examples of this type of *Ir* gene function.

The best explored system that manifests these effects is the genetic control of the immune response to (T,G)-A--L as studied by Edna Mozes and a large number of her colleagues over the past 15 years (Mozes, 1975, 1978). The first intriguing data emerged from the limiting dilution studies (discussed in Section III,B) performed by Lichtenberg *et al.* (1974). For (T,G)-A--L it had been shown that mice bearing the $H\text{-}2^b$ haplotype were high responders, while mice bearing the $H\text{-}2^k$ and $H\text{-}2^s$ haplotypes were low responders.

Interestingly, the limiting dilution analysis suggested that the two low responders were qualitatively different. *H*-2^k-bearing mice manifested only a decreased frequency of bone marrow precursors; their thymocyte limiting dilution curve was indistinguishable from that of the high responder. In contrast *H*-2^s-bearing mice had both a decreased frequency of bone marrow precursors and an altered thymocyte limiting dilution curve. The latter did not conform to the expected Poisson distribution and therefore could not be interpreted as simply a decreased T cell precursor frequency. However, the data definitely suggested that a difference existed in the cooperative potential of thymocytes from low responder *H*-2^s-bearing mice. The data were interpreted at the time as a difference in the recognition of particular determinants on the (T,G)-A--L molecule. Because *H*-2^k-bearing mice could respond to other antigens with an A--L backbone such as (H,G)-A--L and (Phe,G)-A--L, whereas *H*-2^s-bearing mice could not respond to any of these synthetic polypeptides, but could respond to (Phe,G) and (T,G) determinants on a Pro--L backbone, it was concluded that the thymocyte deficiency of *H*-2^s-bearing mice represented a defect in the ability to recognize determinants located in the A--L backbone. In other words, inability to recognize a carrier determinant was correlated with a T cell defect.

Although these early studies are difficult to interpret in light of current knowledge of *Ir* gene function, the factual observations appear to be correct and have been confirmed in a number of other ways. The primary confirmation has come from the study of T cell helper factors (for a review see Germain and Benacerraf, 1980). These were first prepared by Taussig (1974) who transferred nonimmune thymocytes into syngeneic irradiated (850 R) recipients and immunized with antigen the next day. Seven days later the spleen cells from the recipients were restimulated with antigen *in vitro* and the culture supernatants collected after 6–8 hours. These supernatants, when injected into irradiated recipients along with antigen and bone marrow cells, could replace the requirement for thymocytes in the production of an antibody response. The critical factor in these supernatants was derived from T cells, was antigen specific, and acted in consort with allogeneic as well as syngeneic bone marrow cells to generate an antibody response. When the (T,G)-A--L system was examined by Taussig *et al.* (1974) it was found that thymocytes from *H*-2^k-bearing low responder mice, in addition to thymocytes from high responder *H*-2^b-bearing mice, were capable of producing the helper factor. In contrast, thymocytes from *H*-2^s-bearing low responder mice could not produce the factor. These results correlated perfectly with the earlier limiting dilution studies.

Because the factor was not genetically restricted in its interaction with the bone marrow cells (i.e., did not have to be derived from a strain sharing the same MHC), it was possible to test various allogeneic combinations of

the two components. Bone marrow cells from $H\text{-}2^b$-bearing high responder mice synergized with helper factor from either high responder $H\text{-}2^b$-bearing mice or low responder $H\text{-}2^k$-bearing mice. However, bone marrow cells from the low responder $H\text{-}2^k$-bearing mice would not function with helper factor from either source. This result suggested that the low responder bone marrow cells were defective in their ability to receive help. Subsequent studies by Munro *et al.* (1974) and Munro and Taussig (1975) showed that bone marrow cells from $H\text{-}2^k$-bearing mice could not adsorb out the factor from the supernatants the way that bone marrow cells from $H\text{-}2^b$-bearing mice could. These results were originally interpreted as evidence for a B cell defect in $H\text{-}2^k$-bearing mice similar to the conclusion reached from the limiting dilution analysis. However, since bone marrow is a complex mixture of cells including T cells and macrophages in addition to B cells, it is difficult to be certain what the critical cell type really was.

In screening a number of other strains, Munro and Taussig (1975) discovered one strain, the B10.M ($H\text{-}2^f$), whose thymocytes were defective in factor production, but whose bone marrow cells would adsorb and synergize with factors from thymocytes of $H\text{-}2^b$ or $H\text{-}2^k$-bearing mice. In fact, this strain appeared to be the reciprocal of the $H\text{-}2^k$-bearing strain, which produced the factor, but failed to adsorb it. Therefore, Munro and Taussig attempted to see if these two strains would complement by breeding ($H\text{-}2^f \times H\text{-}2^k$)$F_1$ hybrids such as (B10.M×B10.BR)F_1. Initially they reported that complementation did occur and that the F_1 hybrids were high responders to (T,G)-A--L (Munro and Taussig, 1975). Subsequently a number of laboratories were unable to confirm this observation (Marrack and Kappler, 1978) including Munro and Taussig (1977) themselves. The reasons for this are not entirely clear. However, a study by Young *et al.* (1982) found that the demonstration of complementation was highly antigen-dose dependent and that immunization of the F_1s with 50 μg instead of 10 μg of (T,G)-A--L allowed the complementation to be readily seen.

The biochemical nature of the thymocyte-derived helper factors has not been extensively explored (Germain and Benacerraf, 1980). Most of them have molecular masses in the range of 35,000 to 50,000 Da as determined by gel filtration chromatography. All of them are antigen specific as determined by their ability to be bound and eluted from antigen-coated immunoadsorbent columns. The antigen binding moiety appeared to be similar to V_H gene products from the Ig heavy chain locus as the factors could be removed by adsorption on columns containing anti-V_H antibodies (Eshhar *et al.*, 1980) and in some cases columns containing antiidiotypic antibodies directed against site-specific determinants on conventional antibodies specific for (T,G)-A--L (Mozes and Haimovich, 1979). However, it has not been clearly delineated whether IgV_H gene products directly contribute to these factors

or only indirectly influence their composition and specificity during development, through interactions with B lymphocytes and circulating antibody molecules.

The fine specificity of the (T,G)-A--L-specific factor derived from a high responder $H\text{-}2^b$-bearing mouse was found to be different from either anti-(T,G)-A--L antibodies or the T cells involved in a proliferative response (Mozes, 1976). The helper activity was removed by an immunoadsorbent containing (T,G)-A--L, (Phe,G)-A--L, or G-A--L but not (T,G)-Pro--L or A--L. This pattern was different from anti-(T,G)-A--L antibody, which could react with all of these polymers and different from the proliferating T cells studied by Schwartz *et al.* (1977) which only responded to (T,G)-A--L and (Phe,G)-A--L but not G-A--L. These observations suggested that the factor was derived from a different T cell than the one involved in the proliferation assay.

B. "Ia-Like" Molecules Expressed by T Cells

In addition to an antigen-binding specificity, the helper factors appeared to bear determinants encoded for by genes in the *I-A* subregion of the MHC, because the factors could be bound to and eluted from anti-Ia antisera immunoadsorbant columns (Taussig *et al.*, 1975). For a long time it was not clear whether the Ia molecules associated with the factor were made by the T cells that produced the antigen binding moiety or whether the molecules were actually produced by B cells and macrophages and bound to the T cell factor as a consequence of its specificity for Ia molecules. This dilemma was resolved with the development of T cell hybridomas specific for (T,G)-A--L by Eshhar *et al.* (1980). These clonal populations were grown *in vitro* in the absence of macrophages for many months and still they produced factors bearing "Ia-like" molecules. Thus, the T cell must be the source of this component.

However, no biochemical evidence exists which shows that this component is an MHC-encoded Ia molecule. In fact, the possibility that these "Ia-like" molecules might be uniquely expressed by T cells was first suggested by the serological studies of Taussig *et al.* (1975). Anti-Ia antibodies raised against cells expressing the specific allelic products of the *I-A* subregion of the strain from which the factor was derived were capable of adsorbing out the factor. In contrast, anti-Ia antisera containing public specificities capable of cross-reacting with B cell Ia molecules from other strains would not adsorb out the factor derived from these other strains. This suggestion of unique T cell Ia determinants was confirmed by Tokuhisa *et al.* (1978) working with a very similar T cell enhancing factor. KLH primed Lyt 1^+2^- T cells from B10 congenic mice produce a factor (TaF) capable of enhancing the IgG antibody response to DNP-KLH. TaF was found to be antigen specific and

to possess determinants that appeared to be coded for by genes in the *I-A* subregion of the MHC. The latter were detected by adsorption of the factor on anti-Ia immunoadsorbant columns. When these anti-Ia antisera were first adsorbed with B cells and macrophages to remove all their detectable cytotoxic activity for spleen cells, the antisera were still capable of removing TaF activity. In contrast, when the antisera were adsorbed with nylon wool-purified T cells, the ability to subsequently adsorb TaF was eliminated, even though the cytotoxic activity against splenic B cells and macrophages was untouched. These results demonstrated that at least some of the "Ia-like" determinants associated with the T cell enhancing factor were clearly distinct from the Ia determinants found on B cells and macrophages. This conclusion was confirmed with monoclonal antibodies raised against T cell hybridomas producing TaF (Hiramatsu *et al.*, 1982).

C. Evidence for and against MHC-Restricted T Cell–T Cell Interactions

The clear demonstration in the system of Tada and his colleagues that the factor did not replace the need for helper T cells in an antibody response, but rather only enhanced helper activity, raised the possibility that the augmentation was mediated through a T cell–T cell interaction. Evidence consistent with this concept was uncovered in studies of the DTH response to (T,G)-A--L by Strassmann *et al.* (1980a,b). They used an adoptive transfer system in which T cells were primed with (T,G)-A--L *in vitro* or *in vivo* and then irradiated and transferred iv into naive recipients. DTH was measured by challenging the recipient with (T,G)-A--L in one ear, with saline in the other, and measuring the relative uptake of ^{125}IUdR into the site of inflammation. Only mice possessing the $H\text{-}2^b$ haplotype manifested a DTH response. Mice possessing the $H\text{-}2^k$, $H\text{-}2^f$, and $H\text{-}2^s$ haplotypes were nonresponders. F_1 crosses between all nonresponders and the $H\text{-}2^b$-bearing responder strain were responders and responsiveness was controlled by genes mapping in the *K* region or *I-A* subregion. The interesting result, which established a parallel between this DTH system and the previous helper T cell system, was revealed when cells from nonresponder strains were used to transfer DTH to naive (responder×nonresponder)F_1 recipients. T cells from $H\text{-}2^k$-bearing, nonresponder C3H mice could transfer DTH for (T,G)-A--L into (C3H×C3H.SW)F_1 mice ($H\text{-}2^k \times H\text{-}2^b$), but not to C3H or C3H.SW parental strains (the latter presumably because of a histocompatibility barrier). T cells from responder C3H.SW or (C3H.SW×C3H)F_1 mice could transfer DTH to responder C3H.SW or (C3H.SW×C3H)F_1 recipients, but not to nonresponder C3H recipients. Therefore, $H\text{-}2^k$-bearing strains possessed a radio-resistant cell capable of initiating a DTH response on transfer to a semisyngeneic (F_1) responder

animal. In contrast, T cells from mice bearing the nonresponder $H\text{-}2^f$ or $H\text{-}2^s$ haplotypes were unable to transfer a DTH response to $(H\text{-}2^f \times H\text{-}2^b)$ or $(H\text{-}2^s \times H\text{-}2^b)F_1$ mice, respectively. Thus, similar to their ability to produce T cell helper factors, as discussed above, $H\text{-}2^k$-bearing mice were producers of DTH initiator cells (? factor producers), while $H\text{-}2^f$- and $H\text{-}2^s$-bearing mice were not.

These results suggested that two antigen-specific cells were involved in the DTH transfer system, both of which turned out on subsequent analysis to be T lymphocytes (Straussman *et al.*, 1980b). The initiator T cells that transferred the DTH were Lyt 1^+2^- and radio-resistant after priming with antigen, while the effector T cells were Lyt 1^+2^+, radio-sensitive, and did not require antigen priming. The evidence for T cell–T cell interaction was shown in the following way. C3H thymocytes were educated to (T,G)-A--L by priming in syngeneic irradiated recipients. Seven days later the spleen cells were irradiated and transferred into syngeneic nonresponder C3H recipients or semisyngeneic responder $(C3H \times C3H.SW)F_1$ recipients. Only the latter manifested a DTH response to challenge with (T,G)-A--L. However, if the C3H recipients were also given normal $(C3H \times C3H.SW)F_1$ purified T cells along with the primed, irradiated initiator cells, then a DTH response to (T,G)-A--L was detected. These results suggested that the $H\text{-}2^k$-bearing effector T cell population involved in the DTH response to (T,G)-A--L was the site of the *Ir* gene defect, because nonresponsiveness could be overcome by adding responder F_1 T cells and not F_1 antigen-presenting cells. The authors suggested that the interaction between the two T cells was the critical cellular event that was under *Ir* gene control. However, no clear mechanism has been proposed to indicate how this would occur.

If such T cell–T cell interactions can be influenced by *Ir* genes in the DTH response, it also seems possible that the *Ir* gene effects seen in the (T,G)-A--L specific helper cell assays might stem from similar T cell–T cell interactions. Evidence that indirectly suggests that this might be the case came from experiments with radiation-induced bone marrow chimeras (Marrack and Kappler, 1979). The reader will recall (Section III,E,2) that usually when bone marrow cells from nonresponder mice are allowed to mature in an irradiated $(\text{responder} \times \text{nonresponder})F_1$ recipient, the maturing T cells gain the ability to respond to the antigen in the presence of responder antigen-presenting cells. This is the case for (T,G)-A--L specific T cells from $H\text{-}2^k$- or $H\text{-}2^a$-bearing mice maturing in an $(H\text{-}2^k \times H\text{-}2^b)F_1$. However, Marrack and Kappler (1979) discovered that this was not the case for B10.M ($H\text{-}2^f$) bone marrow cells. B10.M(b.m.) → $(B10 \times B10.M)F_1$(irr) chimeras did not provide help for B10 B cells and macrophages to generate a PFC response to TNP-(T,G)-A--L, even when primed to (T,G)-A--L in the presence of high responder B10 or $(B10 \times B10.M)F_1$ macrophages. PFC responses to

TNP-KLH were observed with cells from these animals. These results suggested that in mice possessing the $H\text{-}2^f$ haplotype, the (T,G)-A--L *Ir* gene defect was expressed in the helper T cell population. This defect could represent an intrinsic failure of the B10.M T cells to produce T cell helper factor. On the other hand, it is possible that the nonresponsiveness of the chimeric B10.M T cells indicates that there is a requirement for a T cell–T cell interaction in the generation of a (T,G)-A--L-specific helper response. In this type of model, one T cell serves in the same capacity as an antigen-presenting cell, in that its cell surface expressed "Ia-like" molecules must be recognized in association with antigen in order for the second T cell to be stimulated. Thus, altering the T cell repertoire of the B10.M T cells in the chimera so that they could recognize B10 MHC-encoded molecules would not overcome the failure in T cell–T cell interaction, because the B10.M T cells only express $H\text{-}2^f$-encoded "Ia-like" molecules. To convert the B10.M to a responder would require changing the MHC phenotype of the T cell.

The appealing aspect of such a T cell interaction model is that it allows one to unify *Ir* gene function. Ia and "Ia-like" molecules become signal flags on populations of lymphoid cells ensuring that antigen-specific responses are channeled through appropriate pathways. The repertoire of each T cell subpopulation would be skewed toward the recognition of the particular Ia molecule needed to be recognized on the surface (or factor) of the next (or previous) cell in the pathway. *Ir* gene defects would reflect antigen-specific holes in the repertoire for that pathway, generated by any one of the six mechanisms discussed in Section VI. A similar analysis would hold for suppressor T cell–helper T cell interactions as discussed in Section VI,C,4,e. Thus, T cell–T cell interactions become analogous to T cell–B cell and T cell–antigen-presenting cell interactions.

However, the reader is cautioned that these ideas are purely speculative, based on only a few experiments in the literature. Other rare examples of low responder T cells failing to convert to a high responder phenotype have been reported, for example, in the lysozyme system (Hill and Frelinger, 1982) and in the cytotoxic response to the H-Y antigen (discussed in Section VIII,C,1,b). On the other hand, these experiments are controversial. For example, Erb *et al.* (1980) have demonstrated that $H\text{-}2^s$(b.m.) $\rightarrow$ $(H\text{-}2^s \times H\text{-}2^b)F_1$(irr) chimeras possess T cells capable of responding to (T,G)-A--L in the presence of responder $H\text{-}2^b$ or $(H\text{-}2^s \times H\text{-}2^b)F_1$ macrophages. One would have expected $H\text{-}2^s$-bearing strains to behave like $H\text{-}2^f$-bearing strains, since neither can produce a (T,G)-A--L-specific T cell helper factor. Yet the results obtained in the chimera experiments suggest that T cells from $H\text{-}2^s$-bearing mice do not possess an intrinsic *Ir* gene defect.

In addition, molecular biology studies have only rarely detected mRNA encoding class II molecules in helper or suppressor T cells (Hodes *et al.*, 1984;

Kronenberg and Taniguchi, 1984; R. I. Lechler and R. N. Germain, personal communication). This has led several investigators to speculate that the "Ia-like" molecules on these T cells might be antigen-specific receptors instead of MHC-encoded proteins (Schrader, 1979; Hodes *et al.*, 1984; Sim *et al.*, 1984). In this hypothesis ("internal image" model), the receptors bind free antigen, internalize and process it, and return fragments to the surface. These fragments are then recognized by conventional MHC-restricted T cells in association with the "Ia-like" determinants on the antigen-specific receptor. The problem with this model is that it cannot easily explain the chimera experiments. B10.M T cells are educated in the (B10×B10.M)F_1 thymus to recognize B10 Ia molecules. They then should also be capable of recognizing the B10 "Ia-like" determinants on augmenting T helper cell receptors. To explain their failure to do this, one might further postulate that the development or amplification of the augmenting T cell repertoire is dependent on the presence in the periphery of antigen-presenting cells bearing $H\text{-}2^b$-encoded Ia molecules. But even this might not be sufficient because of the chimera experiments with lysozyme (Hill and Frelinger, 1982). In this case double chimeras were set up of the type, responder B10.A(b.m.) + nonresponder B10(b.m.) → (B10.A×B10)F_1(irr). After antigen priming, nonresponder chimeric cells were isolated by anti-$H\text{-}2^a$ and complement treatment, transferred into irradiated F_1 recipients and boosted. Despite the presence during development of responder antigen-presenting cells, the lymphoid cells were still not capable of producing an antibody response to lysozyme. However, these authors assumed that the irradiated F_1 host would provide sufficient antigen-presenting cells of responder type to boost the T cells and that the subsequent T cell–B cell interactions would not be MHC restricted. Unfortunately, both of these assumptions are probably invalid. Thus, a more complex form of the "internal image" model remains as a possible explanation of the data.

The central question that remains to be answered to distinguish between the two models is whether expression of real Ia molecules occurs in any murine T cells. Although the molecular biology data have so far been mostly negative, a few T cell clones have been described in the mouse that do express B cell-like Ia molecules, both by protein and molecular biology criteria (Koch *et al.*, 1983; Singh *et al.*, 1984; R. I. Lechler and R. N. Germain, personal communication). Furthermore, many human T cells when activated express the same class II molecules as B cells (reviewed by Natali *et al.*, 1982). Therefore, it is possible that the failure to detect class II molecules in many murine T cell hybridomas is a reflection of the instability of these hybrids or a cell cycle dependence of the expression of the Ia molecules. Further experiments are clearly warranted to try and explain the apparent discrepancy between the functional and structural data.

VIII. *Ir* Gene Control of Cytotoxic T Cell Responses

A. Introduction

For a long time it was felt that *Ir* genes were located solely in the *I* region of the MHC. This feeling was rooted so firmly in the thinking of workers in the field that in fact when the first *Ir* gene in a cytotoxic system was mapped to *H-2K*, Schmitt-Verhulst and Shearer (1976) placed the gene to the left of the *I-A* subregion but still within the *I* region. However, if one understands helper cell *Ir* gene effects as the failure to stimulate T cells capable of recognizing the antigen in association with a particular Ia molecule, then it seems reasonable to imagine that other T cells, which utilize different MHC-encoded products as their restriction elements, would manifest *Ir* gene control mapping to the MHC genes encoding the products that they must recognize. In this section we will outline the experiments in several systems that demonstrate that classical cytotoxic T cell *Ir* genes show all of the properties of helper T cell *Ir* genes, but map to the loci encoding class I molecules. In addition, we will discuss three other categories of *Ir* genes, which represent epistatic effects of one MHC locus on another, the end result of which is a state of nonresponsiveness in the cytotoxic limb of the immune response.

B. Classical-Type Cytotoxic *Ir* Genes

1. Biological Parallels with I Region Genes

a. Antigen- and Allele-Specific Defects. The identification of *Ir* genes influencing cytotoxic T cell responses was not made for a long time mainly because of the complexity of the initial systems being studied. In contrast to the synthetic polypeptides used to define *I* region-encoded *Ir* genes, investigators in the cytotoxic field were concerned primarily with viruses, groups of minor histocompatibility antigens or trinitrophenyl (TNP) modification of a large number of self proteins (for comprehensive reviews of this area see Shearer and Schmitt-Verhulst, 1977; Zinkernagel and Doherty, 1979; Henney and Gillis, 1984). In addition, at least 3 different restriction elements, K, D, and L, in the mouse, are involved in the response. This increases the probability that a given foreign antigenic determinant will be recognized by the host's immune system and that a cytotoxic response will be made. Thus, it was not until the workers in the field began to focus on individual restriction elements that they noticed certain blind spots in the cytotoxic T cell response.

Mice bearing the $H\text{-}2^k$ haplotype are responders to vaccinia virus and generate cytotoxic T cells capable of lysing ^{51}Cr-labeled, virally infected, $H\text{-}2^k$-bearing target cells (either tumors or macrophages) (Koszinowski and

Ertl, 1975). However, when Zinkernagel *et al.* (1978a) examined the responding population for specificity for individual restriction elements, they found that there was no killing of targets expressing vaccinia virus in association with D^k (e.g., C3H.OH, K^dD^k). Therefore, the killing of *H*-2^k-bearing targets was solely directed against virus in association with K^k. Further analysis of this finding revealed that all strains possessing D^k failed to generate anti-vaccinia virus specific cytotoxic T cells restricted to D^k. For example, C3H.OH mice, which possess *d* alleles in the *K* and *I* regions but the *k* allele in the *D* region, responded to vaccinia virus by generating cytotoxic T cells specific for the virus in association with K^d and not D^k. Similarly B6.AK1 mice, which possess *b* alleles in the *K* and *I* regions but the *k* allele in the *D* region, responded to vaccinia virus by generating cytotoxic T cells specific for the virus in association with K^b and not D^k. Thus, the selective low responsiveness to vaccinia virus appeared to be an intrinsic property of the D^k allelic product which was uninfluenced by the genes in the *K* or *I* region.

The defect in the D^k allelic product was antigen specific. C3H.OH mice (K^dD^k) immunized with lymphocytic choriomeningitis (LCM) virus generated cytotoxic T cells capable of lysing LCM virus-infected *H*-2^k-bearing targets in addition to LCM virus-infected *H*-2^d-bearing targets. Similarly C3H mice infected with LCM virus generated cytotoxic T cells capable of lysing LCM virus-infected C3H.OH (K^dD^k) macrophage targets. Thus, cytotoxic T cell specific for LCM virus in association with D^k could be generated by these mice, but not T cells specific for vaccinia virus in association with D^k.

In addition to antigen specificity, the defect was also allele specific. The C57BL/6 strain, which possesses the *b* allele in the *D* region, was a responder to vaccinia virus and generated cytotoxic T cells specific for the virus in association with D^b as measured by the killing of virally infected B10.A(2R) ($K^kI^{k/d}D^b$) targets. Similarly, the B10.A(5R) strain ($K^b,I^{b/k},D^d$), which possesses the *d* allele in the *D* region, generated cytotoxic T cells specific for vaccinia virus in association with D^d as well as K^b. Thus, only the D^k allelic product failed to function in association with vaccinia virus. Similar results were obtained for influenza virus in association with K^b (Doherty *et al.*, 1978), Sendai virus in association with D^k (Kurrle *et al.*, 1978) and D^b (de Waal *et al.*, 1983), the H-Y antigen in association with K^b (Simpson and Gordon, 1977), low-dose TNP-modified self antigens in association with D^q (Levy and Shearer, 1980), vesicular stomatitis virus in association with K^k and D^k (Rosenthal and Zinkernagel, 1981), and alpha viruses such as Sindbis virus in association with all K and D molecules tested, except for D^k (Müllbacher and Blanden, 1978).

The analogy between these cytotoxic systems and classical *Ir* genes for

helper T cells should be readily apparent. In the case of the genetic control of the immune response to a synthetic polypeptide such as GA, strains possessing the *p* or *q* allele in the *I-A* subregion of the MHC are nonresponders to the antigen, whereas strains possessing the *b*, or *d* allele are responders. In the case of the genetic control of the cytotoxic response to vaccinia virus, strains possessing the *k* allele in the *D* region of the MHC are nonresponders, whereas strains possessing the *b* or *d* allele are responders. In both systems nonresponsiveness is associated with the inability to detect T cells capable of recognizing the antigen in association with a particular MHC-encoded gene product. Thus, both types of *Ir* gene effects represent limitations in the ability of T cells to recognize antigen in association with particular MHC-encoded molecules. I will refer to this type of *Ir* gene effect as classical.

b. Class I Molecules Are the Ir Gene Products. The proof that in fact the *Ir* gene product in a cytotoxic response is the class I molecule itself and not some closely linked gene product, came from the study of *H-2* mutants. The B6-*H*-2^{bm1}(bm1) mutant strain carries an alteration in the structural gene coding for the K^b molecule (Nathenson *et al.*, 1981). It differs from the congenic B6 gene product by three amino acid substitutions. De Waal *et al.* (1983a) discovered that the cytotoxic response against Sendai virus in B6 mice was limited to T cells with specificity for the virus in association with K^b. Analysis of the bm1 mutation revealed that it was a total nonresponder to this virus, suggesting that the alteration in the K^b molecule had altered the immune response phenotype. A similar observation was made for the response to the Moloney sarcoma virus in association with D^b (Stukart *et al.*, 1982). The B6.C-*H*-2^{bm14}(bm14) mutant, which has an altered D^b molecule, failed to generate a cytotoxic response against the virus, in contrast to the congenic B6 strain. Thus, both the K and D molecules appeared to function as *Ir* gene products. Again, however, one should keep in mind the caveat that the spontaneous MHC mutation may have arisen in a mouse with alterations at other genetic loci. Therefore, the only way to prove that a particular genetic locus controls *Ir* gene function is to transfer the gene, in its normal and mutant forms, to another host and show that the transgenic mouse acquires the Ir phenotype of the donor (see Section V,D).

c. (Responder×Nonresponder)F_1 Crosses. The situation for F_1 crosses between responder and nonresponder strains has not been well studied in cytotoxic systems, but those experiments that have been done suggest that the outcome is similar to that observed with *I* region-encoded *Ir* genes. In the classical *I* region systems, the F_1 cross between a responder and nonresponder is a responder, because the T cells that recognize the antigen in association with responder Ia molecules are not affected by the nonresponder Ia molecules. For a similar reason, the F_1 hybrid does not overcome

the inability to recognize the antigen in association with the nonresponder Ia molecule. This latter point is usually demonstrated in antigen-presentation experiments in which antigen-pulsed nonresponder parental macrophages fail to stimulate a response in primed F_1 T cells.

For classical cytotoxic *Ir* genes the situation concerning the nonresponder MHC-encoded molecules appears to be similar. T cells specific for the antigen in association with the nonresponder class I molecule are not found in the F_1 hybrid. For the cytotoxic response to vaccinia virus (Zinkernagel *et al.*, 1978a), the cross between the D^k-bearing nonresponder, C3H.OH, and the D^b-bearing responder, C57BL/6, did not allow the F_1 hybrid to generate cytotoxic T cells capable of lysing vaccinia virus in association with D^k. Although Zinkernagel *et al.* (1978a) did not test for killing against *H-2*^{b}-bearing targets in this experiment, a subsequent study (Zinkernagel *et al.*, 1978b) with [C3H×C57BL/6]F_1 mice revealed the presence of cytotoxic T cells specific for vaccinia virus in association with D^b, the high responder allelic product. Unfortunately the magnitude of the cytotoxic response in this F_1 combination was greatly reduced because of the immunodominance effect (discussed in Section VIII,C,2).

A cleaner demonstration of the separate effects of cytotoxic *Ir* genes on T cells specific for responder versus nonresponder class I allelic products in F_1 mice was published by de Waal *et al.* (1983a) using an *H-2* mutant. (B6×bm1)F_1 mice represent a cross between a responder and a nonresponder strain for the cytotoxic response to Sendai virus. The F_1 was a responder to Sendai virus, suggesting that responsiveness was dominant. However, the cytotoxic T cells were only specific for Sendai-infected B6 targets and not Sendai-infected bm1 targets. In addition, spleen cells from Sendai virus-primed mice could be stimulated *in vitro* to give a cytotoxic T cell response by Sendai-infected B6 parental stimulator cells but not by Sendai-infected bm1 parental stimulator cells. Again, the killing, when detected, was directed against the virus in association with the B6 MHC-encoded molecule, K^b. Thus, the F_1 could generate cytotoxic T cells specific for the virus in association with the responder allelic form of the class I molecule (K^b) but not T cells specific for the virus in association with the nonresponder allelic form of the class I molecule (K^{bm1}). Similar results were obtained by Stukart *et al.* (1982) for the cytotoxic T cell response to Moloney virus in (B6×bm14)F_1 mice. Thus, in agreement with *I* region *Ir* gene phenomenology, the classical cytotoxic *Ir* genes appear to be codominantly expressed.

d. Nonresponders Fail to Activate a Family of T Cell Clones with Different Fine Specificity. One of the hallmark experiments of *I* region-encoded *Ir* genes was the demonstration by Levine *et al.* (1963a) that nonresponder guinea pigs could not respond to a variety of different haptens on the same carrier molecule (PLL), while responder guinea pigs could discriminate

among these various hapten conjugates (see Section II,B). A similar phenomenon was noted in the cytotoxic T cell response to type A influenza virus. Many different cytotoxic T cell clones could be elicited from BALB/c mice (K^dD^d) in response to immunization with influenza virus (Braciale *et al.*, 1981a). Of those recognizing the virus in association with K^d, at least two and possibly three different phenotypes were distinguished by testing for killing against K^d-bearing targets infected with different influenza virus strains. Similarly, immunization of BALB/c (K^dD^d) and CBA/J (K^kD^k) mice with different viral influenza subtypes revealed the existence of subtype-specific cytotoxic T cells (Effros *et al.*, 1977; Braciale, 1979). In contrast, the nonresponder K^b allele in B10.A(5R) mice (K^bD^d) was not recognized at all in association with any influenza virus determinants or subtypes tested (Doherty *et al.*, 1978). This was an allele specific defect that was not a consequence of a lack of helper T cells, because both the BALB/c and B10.A(5R) strains made comparable cytotoxic responses to influenza virus in association with D^d. Thus, the responder strains possessed many different potentially responding cytotoxic T cell clones specific for the virus in association with K^d or K^k while the low responder strain had few or no cytotoxic T cells specific for the virus in association with K^b. These results are consistent with the existence of an "agretope" on the viral antigen, a site that interacts with K^k or K^d but not K^b during the activation of the T cells (see Section VI,B,2,c).

e. Chimera Experiments. The final analogy between classical helper and cytotoxic *Ir* genes was demonstrated in the studies with radiation-induced bone marrow chimeras. When bone marrow from an *I* region nonresponder animal is allowed to mature in a responder environment, the resulting T cells are usually capable of responding to the antigen, but only in association with responder Ia molecules (see Section III,E). An analogous result was obtained for cytotoxic *Ir* genes. Zinkernagel *et al.* (1978b) set up irradiation chimeras of the type, C3H.OL($K^dI^dD^k$)(b.m.) → B10.D2($K^dI^dD^d$)(irr). After 3 months the chimeric T cells were immunized with vaccinia virus in an acutely irradiated, virally infected B10.D2($K^dI^dD^d$) host in order to provide antigen-presenting cells bearing D^d. The resulting cytotoxic T cell population was capable of lysing vaccinia virus infected targets expressing D^d [B10.A(5R):$K^bI^{b/a}D^d$]. Thus, cytotoxic T cells bearing the nonresponder D^k allele had acquired the ability to recognize vaccinia virus in association with the responder allelic product, D^d, by maturing in an environment expressing this product. The failure to acquire responsiveness to vaccinia virus in association with D^k was not shown in this experiment because the priming environment of the B10.D2 host did not contain any antigen-presenting cells bearing D^k. A similar problem prevented the interpretation of experiments on the role of the thymus and the K^b allele in the generation of autoimmune

thyroiditis, a supposed cytotoxic T cell-mediated disease (Maron and Cohen, 1980).

The reciprocal chimera experiment, in which bone marrow from a responder animal was allowed to mature in a nonresponder environment, was also carried out by Zinkernagel *et al.* (1978b) but insufficient controls were performed to interpret the results. Spleen cells from BALB/c($K^dI^dD^d$)(b.m.) → C3H.OH($K^dI^dD^k$)(irr) chimeras were primed to vaccinia virus in (BALB/c ×C3H.OH)F_1 irradiated recipients and tested for cytotoxic activity on virus infected H-2^k- and H-2^d-bearing targets. No killing of H-2^k-bearing targets was observed, suggesting that a "responder" population that has "learned" to recognize D^k as a restriction element (presumed but not shown for this set of chimeras) did not circumvent the inability to recognize vaccinia virus in association with D^k. On the other hand, the spleen cells did lyse H-2^d-bearing targets infected with vaccinia virus. This result at first seems surprising, as it suggests that the donor population did not lose the ability to respond to vaccinia virus in association with D^d (the expected result from allogeneic chimeras for helper cell *Ir* genes, see Table II). However, the result probably reflects the greater complexity inherent in the cytotoxic response. Mice bearing the H-2^d haplotype have responder alleles at both the *K* and *D* loci. Thus, the response detected from the chimeric spleen cell population could have been specific for vaccinia virus in association with K^d. To rule this in or out one would have to examine the responding population for cytotoxicity against vaccinia virus-infected recombinant targets such as B10.A(5R) (K^bD^d) and C3H.OH (K^dD^k). Since this was not done, no conclusions could be reached.

The responder(b.m.) → nonresponder(irr) chimera experiment was unambiguously carried out in a different cytotoxic system, the response to the male-specific, minor histocompatibility antigen, H-Y. Females of the C57BL/10 (B10) strain, when immunized and boosted with B10 male spleen cells, generated cytotoxic T cells specific for the H-Y antigen in association with D^b but not K^b (Simpson and Gordon, 1977). In contrast, the recombinant strain B10.A(5R) ($K^bI^{b/a}D^d$) was a nonresponder to H-Y (von Boehmer *et al.*, 1978). This was assumed to be because D^d is a nonresponder allele, although the B10.A(5R) has an additional Ia molecule (E^b_β:E^k_α), which through a suppression mechanism (see Section VI,C,4,e) could eliminate T cell help. However, the nonresponder D^d allele assumption is probably correct since (B10×BALB)F_1 females primed and boosted with BALB/c male spleen cells generated cytotoxic T cells specific for H-Y in association with K^d and not D^d (Simpson and Gordon, 1977). When von Boehmer *et al.* (1978) set up female (B6×CBA)F_1(b.m.) → female B10.A(5R)(irr) chimeras and immunized them with (B6×CBA)F_1 male cells, they failed to develop a cytotoxic response against the H-Y antigen. In contrast, female (B6×CBA)F_1(b.m.) → female

B6(irr) chimeras, when immunized with F_1 male spleen cells, were capable of generating a cytotoxic T cell response against the H-Y antigen in association with *H*-2^b-encoded MHC gene products (presumably D^b). Thus, female T cells bearing the responder D^b allele, primed with male cells bearing the D^b gene product were only capable of generating cytotoxic T cells if they had matured in a thymic environment expressing D^b. If the developmental environment of the irradiated host (presumably cells in the thymus based on analogy to helper T cell *Ir* genes) expressed the product of the nonresponder D^d allele, then no T cells emerged capable of recognizing the H-Y antigen in association with D^b and the animal was a nonresponder. These results demonstrated that the cytotoxic Ir phenotype was an acquired characteristic, analogous to what had been shown for the *I* region-encoded Ir phenotype.

One possible exception to this conclusion has recently been reported by Kast *et al.* (1984). Athymic *H*-2^b-bearing nude mice were grafted with either B6 (responder to Sendai virus) or bm1 (nonresponder to Sendai virus) neonatal thymuses and immunized with Sendai virus 2–3 months later. Both chimeras gave a cytotoxic T cell response to Sendai virus in association with K^b and not K^{bm1}. Thus, the presence of a nonresponder thymic developmental environment did not preclude the ability to respond to Sendai virus. The authors interpreted this failure in terms of an extrathymic repertoire development, i.e., that cytotoxic T cell precursors specific for Sendai virus and K^b molecules somehow differentiated outside the thymus in peripheral sites expressing responder K^b molecules. However, the nude mouse is known to express a thymic rudiment early in development (Jenkinson *et al.*, 1981) and the possibility that the cytotoxic T cell precursors differentiated in this organ before it involuted was not eliminated (see R. Schwartz, 1984, for a review of this problem).

Overall the biological data suggest that classical-type cytotoxic *Ir* genes are functionally equivalent to *I* region-encoded *Ir* genes. They represent an intrinsic property of an MHC-encoded class I molecule which affects in an antigen-specific way the cytotoxic T cell response. The discovery of these effects brought to completion the unification of T cell immunology by demonstrating that the two major classes of MHC-restricted T cells both had antigen- and MHC allele-specific blind spots in their responses.

2. *Structural Parallels with I Region Genes: A Comparison of Class I and Class II Molecules and Genes*

More recently this unification has been completed at the molecular level as well. A structural comparison between class I and class II molecules has revealed some unexpected parallels (see Fig. 5). At the protein level (see Kaufman *et al.*, 1984, for a review) the Ia molecules consist of two transmembrane glycoproteins each with two external domains. The α chain has

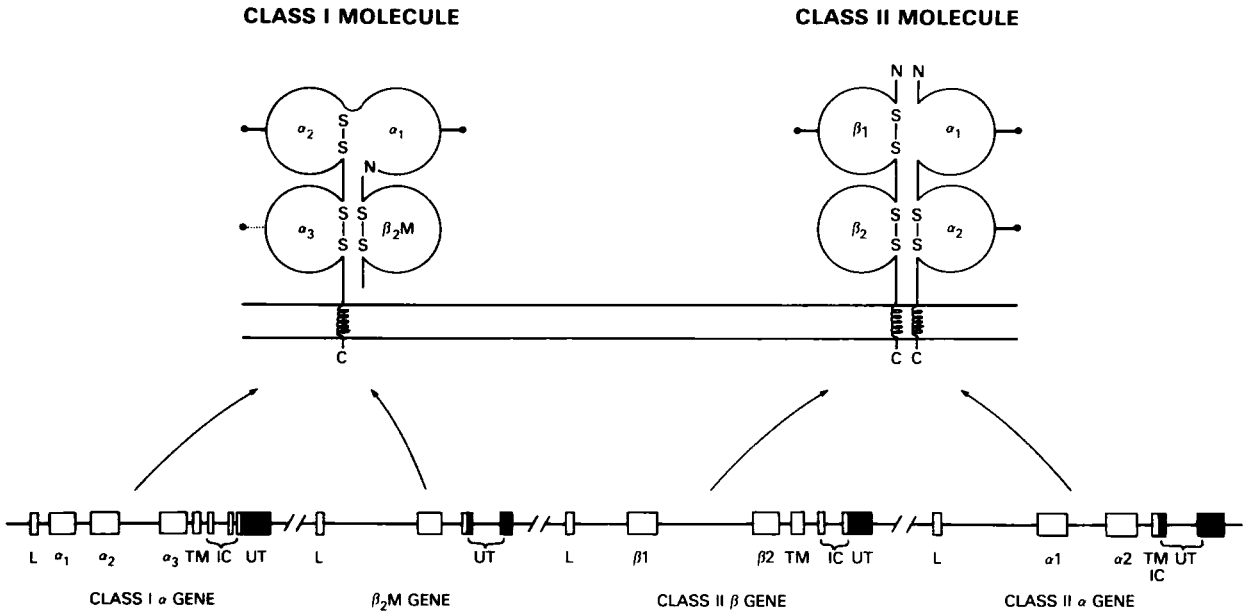


FIG. 5. Structural homologies between MHC class I and class II molecules and genes. In the genomic organization of the four genes shown, each rectangle represents an exon and the lines between them introns. The clear rectangles represent coding sequences and the shaded exons or parts of exons represent untranslated segments (UT). L encodes the leader peptide; α_1, α_2, α_3, β_1, and β_2 encode the extracellular domains of the proteins; TM encodes the transmembrane portion; and IC encodes the intracytoplasmic portion. The β_1-microglobulin gene is located on chromosome 2; the other 3 genes are located in the MHC on chromosome 17. In the protein structures shown, N and C indicate the N-terminal and C-terminal ends of the protein. Each half circle connected by an S–S represents an extracellular disulfide-bonded domain of the protein. The α_1 domains are shown in the same configuration, although they do not contain an S–S bond. The spiral through the membrane represents the proposed alpha helical structure of the transmembrane portion of the protein. The solid black pins extending from the extracellular domains of the proteins represent N-linked complex carbohydrate groups. The one pin made up of dotted lines indicates that this domain (α_3) is variably glycosylated depending on the particular allelic form of the protein. This figure is based on data from the reviews by Hood *et al.* (1983) and Kaufman *et al.*

an apparent molecular mass of 33–35 kDa and the β chain an apparent molecular mass of 27–29 kDa. Both domains of the β chain contain an intrachain disulfide bond, β_1 having cysteines spaced by 63 amino acids and β_2 having cysteines spaced by 55 amino acids. In the α chain only the α_2 domain contains an intrachain disulfide bond and it has a spacing of 55 amino acids, similar to that of the β_2 domain. At the molecular biology level (see Hood *et al.*, 1983, for a review) the class II α and β genes also show characteristic structures. The leader sequence of the α gene is separated from the segment encoding the first domain (α_1) by a large intron, and the 3′ untranslated segment is split in an unusual manner by an intron. The β gene contains neither of these features, but has a large intron between the first and second domains, and multiple exons making up the coding regions for the transmembrane and intracytoplasmic portion of the molecule. Exon shuffling experiments, in which different allelic forms of the exons encoding the first and second domains of the β chain have been switched and the resulting hybrid genes expressed by transfection into L cells (Germain *et al.*, 1985), have revealed that T cells recognize primarily the allelic polymorphisms in the β_1 domain.

Class I molecules initially appeared to be a separate group of proteins, consisting of one 44 kDa transmembrane glycoprotein noncovalently associated on the cell surface with β_2-microglobulin, a 12 kDa protein encoded outside the MHC (on chromosome 2). However, as more structural detail became available, some intriguing parallels with class II molecules began to emerge. β_2-Microglobulin (β_2m) is a polypeptide composed of 99 amino acids, which contains an intrachain disulfide bond of cysteine residues separated by 54 amino acids. β_2m shows significant amino acid sequence homology to the α_2 and β_2 domains of class II molecules as well as the constant and variable regions of immunoglobulin. At the DNA level, the gene encoding β_2m (Parnes and Seidman, 1982) shows a genomic organization that has two features in common with the class II α gene: the long intron between the leader sequence and the first domain-encoding exon (α_1) and the unusual intron splitting of the 3′ untranslated segment. In contrast, the DNA organization of the MHC-encoded 44 kDa molecule shows structural features more akin to the class II β gene: the transmembrane and intracytoplasmic domains are encoded by multiple exons and the largest intron occurs between the exons encoding the second (α_2) and third (α_3) domains. Exon shuffling experiments (reviewed in Burakoff *et al.*, 1984) have shown that the correct allelic forms of both the α_1 and α_2 external domains are necessary for T cell recognition, but not the correct allelic form of the α_3 domain. Thus, the large intron corresponds to the functional separation in the molecule.

At the protein level, the three external domains of the class I 44 kDa molecule are not all structurally similar. The two membrane proximal do-

mains (α_2 and α_3) contain intrachain disulfide bonds whereas the N-terminal α1 domain does not. The α_3 domain shows the greatest amino acid sequence homology to β_2m and the α_2 and β_2 domains of class II molecules. The intrachain disulfide bond has its cysteines separated by 55 amino acids. In contrast, the α_2 domain shows a greater homology to the β_1 domain of the class II molecule with an intrachain disulfide bond containing cysteines separated by 62 amino acids. Finally, the lack of a disulfide bond in the N-terminal α1 domain is homologous to the α_1 domain of the class II α chain. Both also have a single complex N-linked carbohydrate, although the class II α_1 domain is consistently smaller (85–88 amino acids) than the class I α_1 domain (~90 amino acids).

These hints of a structural similarity between class I and class II molecules have led to the parallel models shown in Fig. 5. β_2m has been shown to bind to the α3 domain of the 44 kDa class I molecule (Yokoyama and Nathanson, 1983). These two domains are homologous to the α_2 and β_2 domains of the class II molecules and thus have been pictured as forming the two membrane proximal domains in the class I molecule structure. Then, since the α_2 domain of the 44 kDa protein appears homologous to the β_1 domain of the class II molecule, while the α_1 domain appears homologous to the class II α_1 domain, bending the 44 kDa molecule to place the α1 domain over the β_2m gives a class I molecule with similar structure to that of a class II molecule. Preliminary X-ray structural analysis of crystals from a papain cleaved extracellular portion of two human class I molecules (HLA-A2 and HLA-A28) has revealed a 2-fold rotational axis of symmetry, consistent with the general model shown in Fig. 5 (Bjorkman, Strominger, and Wiley, unpublished observations).

This apparent similarity in structure of the class I and class II molecules is also supported by the recent findings on the molecular biology of the T cell receptor for antigen. Both the α and β genes encoding this two polypeptide structure are expressed in both helper and cytotoxic T cells (Davis *et al.*, 1984; Mak and Yanagi, 1984; Chien *et al.*, 1984; Saito *et al.*, 1984). In addition, one particular V_β gene has been found to be expressed in both a D^d-specific alloreactive cytotoxic T cell clone (V. D. Starz, M. J. Bevan, and S. M. Hedrick, personal communication) and a chicken red blood cell-specific helper T cell clone (Patten *et al.*, 1984). If similar findings are made for the V_α genes, then it would appear that the same germ line repertoires are giving rise to the specificities of both T cell subpopulations. This seems reasonable if the structures of class I and class II molecules, which have to be recognized by these repertoires, are very similar. It is also supported by functional experiments with alloreactive cells that have demonstrated the existence of cytotoxic T cells specific for class II molecules (Nabholz *et al.*, 1975; Wagner *et al.*, 1975; de Waal *et al.*, 1981) and helper T cells specific for class I molecules (Swain and Panfili, 1979; Epstein and Cohn, 1978).

Nonetheless, the immune system at some point in development appears to make a distinction between cytotoxic and helper T cells (Cohn and Langman, 1982). If both subpopulations derive their receptors from the same pool, then differentiation must entail a selection step based on specificity for and/or activation by class I versus class II molecules. This would presumably occur in an organ like the thymus in which the two classes of molecules would be differentially expressed on certain cells in such a way that recognition by the immature T cell with a particular affinity would lead to differentiation along one particular developmental pathway. Alternatively, the repertoires might start out the same, but through differential expansion in the periphery, as a consequence of differences in the signaling mechanisms for the two classes of molecules, a functional dichotomy would emerge. Thus, although the overall functional and structural features of class I and class II molecules are grossly similar (presumably because of their common evolutionary origin) the fine details of the structures themselves, the cells on which they are expressed, or the mechanisms by which they activate T cells must be different to allow for the distinct roles they appear to play in the immune system.

3. *Models of Class I Ir Gene Function*

a. Models. The mechanisms by which class I molecules influence the cytotoxic T cell response in an antigen-specific manner have not been extensively explored. Since these molecules are functionally and structurally analogous to class II molecules, one presumes that many of the mechanisms discussed in Section VI will apply (see Table III). Certainly if the germline repertoires are identical, then limitations on the number of receptors could produce holes in the cytotoxic repertoire. Note, however, that the absence of a particular V region gene would have different consequences for cytotoxic and helper T cell recognition. A receptor of value in recognizing a particular antigen in association with a class I molecule might be of no value in recognizing that same antigen in association with a class II molecule.

In fact, it is not yet clear whether a cytotoxic T cell and a helper T cell can recognize the same determinant on the antigen. The two systems seem to focus on different types of antigens. Cytotoxic T cells appear to recognize mainly integral membrane proteins such as viral polypeptides and minor histocompatibility antigens (reviewed in Burakoff, 1981). They almost never respond to soluble protein antigens (Schirrmacher *et al.*, 1974). The one notable exception is TNP-derivatized bovine γ-globulin or bovine serum albumin described by Schmidt-Verhulst *et al.* (1978). In contrast, helper T cells readily recognize soluble protein antigens and, in fact, appear to be specific for small peptide fragments of these molecules (reviewed in Goodman and Sercarz, 1983). This dichotomy has led some investigators to suggest that cytotoxic T cells can only respond to unprocessed antigens, whereas

helper T cells can only respond to processed antigen. This seems unlikely to be true given the probable overlap of the two T cell repertoires. However, the basis for the dichotomy remains unexplained. The possibility that the helper and proliferative T cell responses studied so far represent a skewed sampling of their repertoire because of the way that antigen is administered (e.g., in complete Freund's adjuvant) has not been eliminated. On the other hand, no good studies have been performed to eliminate the possibility that cytotoxic T cells are recognizing antigen fragments. In fact, several studies suggest the opposite is true. For example, in the cytotoxic T cell response to influenza virus, the major cross-reactive specificity appears to be directed against a class I molecule in association with a nuclear viral protein (NP) which cannot be detected on the cell surface in an intact form (Townsend *et al.*, 1984). In addition, cytotoxic T cells specific for the viral hemagglutinin were capable of being stimulated by cyanogen bromide fragments of the molecule (Wabuke-Bunoti *et al.*, 1981). Thus, whether antigen processing will limit the potential response of the cytotoxic T cell repertoire remains to be determined.

Somatic effects on the repertoire, such as tolerance induction and positive selection in the thymus, are additional ways in which class I molecules could influence the cytotoxic T cell repertoire. As discussed in Section VI,C,2 there is some evidence from studies on preferential killing in F_1 mice (Mülbacher *et al.*, 1981; Vucak *et al.*, 1983), for the existence of a clonal elimination mechanism based on tolerance induction. In these cases, cytotoxic T cells specific for the H-Y antigen in association with one class I molecule (e.g., K^b) were shown to be alloreactive against a second class I molecule (e.g., K^s). In an F_1 cross between the two strains expressing these class I molecules ($K^b \times K^s$), the clones specific for the H-Y antigen in association with the first class I molecule were shown to be missing, presumably because they were deleted by a tolerance mechanism. However, whether such a mechanism operates in an inbred nonresponder strain remains to be determined (see Section VI,C,2,c).

Evidence for positive selection comes exclusively from the chimera experiments, as was the case for *I* region-encoded *Ir* genes. How strong a force this will be in shaping the cytotoxic T cell repertoire remains to be determined, since so few experiments have been done. In this regard it should be pointed out that an alternative explanation to the extrathymic repertoire interpretation of the Kast *et al.* (1984) experiments discussed in Section VIII,B,1 is that the bm1 thymus allows for the positive selection of clones specific for Sendai virus in association with K^b. If antigen-presentation effects are the dominant reason for nonresponsiveness in this case, then the bm1 would be a nonresponder, while the bm1(Thy) → B6(nu) chimera would be a responder because it has the right (responder) antigen-presenting cells.

The most extensively studied mechanism of *Ir* gene control involving class I molecules has been antigen presentation. This came about because of the original competing theories put forth to explain the dual specificity of cytotoxic T cells for antigen and class I molecules (reviewed in Zinkernagel and Doherty, 1979). Early investigators argued that either the T cell had two independent receptors, one for antigen and one for the MHC-encoded molecule, or a single receptor that recognized a unique complex of these two ligands. This latter model was referred to originally as the altered self model, when it was imagined that the antigen altered an MHC-encoded cell interaction molecule, and subsequently as the neo-antigenic determinant (NAD) or complex antigenic determinant (CAD) model, when it was thought that a complex of the two ligands was being recognized by the T cell receptor. As a consequence of this theoretical perspective, scientists in the field became involved in experiments to determine whether or not antigen and class I molecules formed complexes on the surface of target cells. From these studies have emerged a few hints that the interaction between viral antigens and class I molecules might be selective.

b. Evidence for a Selective Physical Association between Viral Antigens and Class I Molecules. The first experiment to suggest a physical association between class I molecules and viral glycoproteins was carried out by Schrader *et al.* (1975). EL-4, a C57BL/6 tumor, expresses on its surface the viral gp70 protein of an endogenous murine leukemia virus. Schrader showed that antisera directed against the MHC-encoded molecules of the *H-2*^{b} haplotype would co-cap the viral gp70 protein in addition to the K^b and D^b class I molecules. In the reciprocal experiment, Henning *et al.* (1976) showed that antibodies directed against the viral proteins would co-cap the K^b and D^b class I molecules. These results suggested that a stable interaction existed in the cell membrane between the viral glycoprotein and the class I molecules. When Geiger *et al.* (1979) extended this type of study to vesicular stomatitis virus (VSV), a degree of specificity in the association was detected. Antiviral antibodies co-capped K^b molecules, but not D^b molecules, from the surface of VSV-infected EL-4 cells. This observation correlated with the preferential specificity of VSV-specific cytotoxic T cells obtained from two different *H-2*^{b}-bearing strains, i.e., these cytotoxic T cells showed at least a 10-fold greater lytic activity against VSV-infected target cells expressing only K^b than against VSV-infected target cells expressing only D^b. However, in contrast to the studies of Schrader *et al.* (1975) antibodies against the K^b molecule did not co-cap the VSV antigens. Furthermore, anti-VSV antibodies did not co-cap K^d and D^d molecules on VSV-infected *H-2*^{d}-expressing tumor cells; yet VSV-specific cytotoxic T cells could be generated in *H-2*^{d}-bearing mice with specificity for the virus in association with D region-encoded molecules (now known to be L^d). Thus, the correlation between responsiveness to VSV in association with particular

class I molecules and the ability of these same class I molecules to interact with VSV viral proteins in co-capping experiments was not very extensive.

Another system suggesting a selective association between viral proteins and particular class I molecules was the response to the Friend murine leukemia virus studied by Bubbers *et al.* (1977). BALB.B (*H*-2^b) mice are high responders to this virus in that they readily reject Friend virus-infected syngeneic tumors by generating cytotoxic T lymphocytes specific for the virus in association with *H*-2^b-encoded class I molecules. By testing for cytotoxicity against different virus-infected target cells and by blocking this cytotoxicity with different anti-H-2 sera, Bubbers *et al.* (1977) showed that only the D^b molecule was being recognized in association with the virus. When co-capping experiments were performed, antiviral antibodies partially co-capped D^b molecules, but did not co-cap K^b molecules. Thus, again, there was a correlation between function and possible physical association. However, also again, the correlation was not extensive. For example, antiviral antibodies partially co-capped D^d molecules even though cytotoxic T cells specific for the virus in association with D^d were never elicited.

Perhaps of more significance were the subsequent findings from their laboratory on the incorporation of *H*-2-encoded molecules into mature virions (Bubbers *et al.*, 1978). Type C viruses, such as Friend virus, mature by budding off from the cell surface. Thus, their envelop could potentially contain host membrane proteins in addition to viral glycoproteins. Interestingly, Bubbers *et al.* (1978) discovered that class I molecules were selectively incorporated into mature virions. In a study of 6 different K and D molecules, only D^b and K^k were found to be incorporated. These results were consistent with the idea that these particular class I molecules selectively associated with the viral glycoproteins and thus were incorporated into the mature virion during the budding process. However, one puzzling feature of these experiments was that the virions had to be disrupted with detergent in order to reveal the presence of the class I molecules (which was measured in an assay in which the lysates were used to inhibit the cytotoxic activity of anti-H-2 antibodies). One might have expected that the class I molecules would have been incorporated into the envelope of the virus and thus fully exposed to the external environment without the need for detergent. Nonetheless, the selective incorporation of the D^b molecule over the K^b molecule did correlate with the cytotoxic T cell preference.

Overall, the studies on virus-specific cytotoxic T lymphocytes are highly suggestive of a selective association between particular viral antigens and particular class I molecules, a necessary prerequisite for any type of antigen-presentation model. However, like the studies on class II molecules, convincing direct experimental evidence (e.g., chemical cross-linking experiments) demonstrating a physical interaction between the *Ir* gene product and the antigen has yet to emerge.

C. Epistatic Effects

1. The Cytotoxic Response to the H-Y Antigen

a. T Cell Help in Cytotoxic Responses. Epistasis is a genetic term meaning the effect of the expression of one gene on the functional expression of a second nonallelic gene. Epistatic effects occur among genes in the MHC in a variety of different circumstances. We have already discussed one example for *I*-region-encoded *Ir* genes in the section on suppression (Section VI,C,4,e). In that example, the presence of an $I\text{-}E^k$ subregion gene product led to the formation of LDH_B-specific suppressor T cells that masked LDH_B-specific helper T cells restricted to recognizing the antigen in association with an $I\text{-}A^k$ subregion gene product. In this section we will discuss three examples in the cytotoxic system, two in which an *I* region gene product influences a *K*/*D*-restricted response and the third in which a *K* gene product influences a *D*-restricted response.

The early studies of Cantor and Boyse (1976) on T cell interactions in the generation of a cytotoxic response against foreign antigens demonstrated that Lyt 1^+2^- helper T cells recognizing the antigen in association with *I* region gene products were required in order for Lyt 1^-2^+ cytotoxic T cells to manifest a full response to the antigen in association with *K*/*D* region gene products. Similar helper activity is required for the generation of cytotoxic T cells specific for viral antigens (Ashman and Müllbacher, 1979), tumor-specific antigens (Hamaoka *et al.*, 1979), and TNP-modified syngeneic spleen cells (Finberg *et al.*, 1979; Kruisbeek *et al.*, 1983).

The usual model for T cell help pictures the activated helper T cell releasing lymphokines such as interleukin 2 (IL-2) and cytotoxic T cell differentiation factor (CDF), which allows any cytotoxic T cell precursor that has its antigen-specific receptor engaged to expand and differentiate into an effector cytotoxic T cell (see Nabholz and MacDonald, 1983). In this model there is no physical interaction between the helper T cell and the cytotoxic T cell, although the two cells may have to be in close proximity if the lymphokines are rapidly degraded *in vivo*. In apparent contradiction to this model, however, a few experimental systems described in the literature have suggested that under some circumstances there might be a requirement for an MHC-restricted interaction between these two T cell types (Zinkernagel *et al.*, 1978c; von Boehmer *et al.*, 1978).

b. T Cell Help in the Cytotoxic Response to H-Y. The best evidence favoring the requirement for *I*-region restricted T helper cells comes from studies on the H-Y cytotoxic system by Von Boehmer and Haas (1979). As discussed in Section VIII,B,1,e, the B6 strain ($K^bI^bD^b$) is a responder to the male antigen, generating cytotoxic T cells specific for H-Y in association with D^b, whereas the B10.A(5R) strain ($K^bI^{b/a}D^d$) is a nonresponder to this antigen because both K^b and D^d are low responder alleles. The CBA strain

($K^kI^kD^k$) is also a low responder to the H-Y antigen. However, von Boehmer *et al.* (1977) discovered that this was not because K^k and D^k were nonresponder alleles. If one made an F_1 cross between B6 and CBA, the hybrid was capable of generating a cytotoxic response against male targets bearing $H\text{-}2^k$-encoded class I molecules. Simpson and Gordon (1977) showed that the cytotoxicity was predominantly directed against H-Y in association with D^k, although K^k could also serve as a restriction element when the D allele was a low responder form, i.e., in a (B10×A/J)F_1 hybrid bearing K^k and D^d. From these observations, von Boehmer *et al.* (1978) speculated that the CBA mouse must have an *I* region-associated *Ir* gene defect, i.e., that I^k is a nonresponder allele for generating helper T cells specific for the H-Y antigen.

Experimental support for the concept of an *I* region-restricted helper T cell came from several studies. First, as mentioned in Section V,D,1, the B6.C-$H\text{-}2^{bm12}$ mutant strain was shown by Michaelides *et al.* (1981) to be a nonresponder to the H-Y antigen. This animal has an altered β chain of the Ia molecule encoded in the *I-A* subregion (A_β). Because male cells from this strain would serve as target cells in a cytotoxic assay involving sensitized T cells from the congenic B6 strain, the results demonstrated that the D^b molecule was unaltered in the bm12 and that the mutation had converted only the responder A^b_β:A^b_α Ia molecule to a nonresponder molecule. This conclusion was supported by the failure of de Waal *et al.* (1983b) to detect a T cell proliferative response to the H-Y antigen in primed spleen cells from bm12 mice. These results suggest that an *I* region-restricted T cell response is required to generate an anti-H-Y cytotoxic response.

The third experiment, performed by von Boehmer and Haas (1979) involved the use of radiation-induced bone marrow chimeras of the P_1(b.m.)→ F_1(irr) type. As discussed in Section VIII,B,1,e, nonresponder bone marrow, in this case from B10.A(5R), maturing in a responder host, (CBA×B6)F_1, acquired the ability to respond to H-Y antigen in association with both $H\text{-}2^k$-bearing and $H\text{-}2^b$-bearing cells. The acquisition of responsiveness by the B10.A(5R) T cells reflected the ability to overcome a classical *Ir* gene defect in a chimera by acquiring the ability to recognize the antigen in association with the products of the responder alleles. Furthermore, the ability to recognize the $H\text{-}2^k$-encoded class I molecules confirmed the fact that K^k and D^k were responder alleles. In contrast, a very different result was obtained when nonresponder CBA bone marrow cells were allowed to mature in the (CBA×B6)F_1 responder host. These chimeras did *not* respond to the H-Y antigen, even though it was introduced to the animal on responder (CBA×B6)F_1 spleen cells [recall from Section III,E the requirement for responder antigen-presenting cells in order to see a response in a P_{NR}(b.m.) → F_{1R}(irr) chimera].

To be certain that a limitation in antigen-presenting cells was not responsible for the failure to respond to H-Y, von Boehmer and Haas (1979) also set up female double bone marrow chimeras of the type: B6 ($K^bI^bD^b$)(b.m.) + CBA ($K^kI^kD^k$)(b.m.) → (B6×CBA)F_1(irr). After 2 months, when it was determined that 60% of the lymphocytes expressed H-2^k antigens and 40% H-2^b antigens, the chimeras were immunized with (B6×CBA)F_1 male cells, boosted *in vitro*, and tested for cytotoxicity against CBA (K^kD^k) male targets. All of the cytotoxic activity could be eliminated by pretreating the cells *in vitro* with anti-H-2^b serum and complement. Treatment with anti-H-2^k serum had no effect. In contrast, an alloreactive cytotoxic response against DBA/2 (*H*-2^d) targets could only be eliminated by treating with both anti-H-2^b and anti-H-2^k sera. These results demonstrated that H-2^k-bearing T cells were functionally present in these chimeras (allogeneic control), but that despite the presence of responder antigen-presenting cells in the priming environment (derived from the B6 bone marrow cells), the CBA T cells were not stimulated to generate a cytotoxic response against the H-Y antigen.

To explain this puzzling finding, von Boehmer *et al.* (1978) postulated a requirement for an *I* region-restricted interaction between an H-Y specific helper T cell and an H-Y specific precytotoxic T cell in order to generate the cytotoxic response. In this model, the helper T cell first responds to the H-Y antigen in association with the A^b_β:A^b_α Ia molecule on an antigen-presenting cell. T cells specific for H-Y in association with A^k_β:A^k_α are not activated or do not exist. The helper T cell then provides a differentiation signal to the precytotoxic T cell by recognizing H-Y in association with an "Ia^b-like" molecule expressed on the precytotoxic T cell surface. This signal, in conjunction with the recognition by the precytotoxic T cell's receptor of H-Y in association with D^k, K^k, or D^b, leads to activation of the cell. In the context of this model, the explanation of the nonresponsiveness of the CBA(b.m.) → (CBA×B6)F_1(irr) chimera is as follows. CBA helper T cells differentiating in the (CBA×B6)F_1 thymus acquire the ability to recognize A^b_β:A^b_α Ia molecules. However, this capability is useless as the precytotoxic T cells derived from the donor marrow express on their surface only "Ia^k-like" molecules. Thus, the required T cell–T cell interaction cannot occur and the chimera is a nonresponder to H-Y.

In order to test this T cell–T cell interaction model, von Boehmer and Haas (1979) designed an elegant experiment using a mixture of cells from different types of radiation-induced bone marrow chimeras. Recall that (B6×CBA)F_1(b.m.) → B10.A(5R)(irr) chimeras are nonresponders to the H-Y antigen because they can recognize the products of only K^b and D^d, two nonresponder alleles. Theoretically, however, they should be capable of generating helper T cells specific for the H-Y antigen since they can recog-

nize A^b_β:A^b_α Ia molecules. (B6×CBA)F_1(b.m.) → CBA(irr) chimeras were also shown to be nonresponders to the H-Y antigen. In this case the nonresponsiveness was postulated to result from the failure to generate helper T cells in association with A^b_β:A^b_α Ia molecules, because the chimeric F_1 T cells have their repertoires contracted, i.e., they recognize antigens only in association with *H-2*^{k}-encoded molecules. Von Boehmer and Haas reasoned that if one were to mix the T cells from these two different F_1(b.m.) → P_1(irr) chimeras, the resulting population should respond to H-Y, because the competent (B6×CBA)F_1(b.m.) → B10.A(5R)(irr) helper T cells should provide help for the competent (B6×CBA)F_1(b.m.) → CBA(irr) H-Y-specific precytotoxic T cells, since the latter express "Ia^b-like" molecules on their surface.

This prediction was verified experimentally. Spleen cells from (B6×CBA)(b.m.) → CBA(irr) female chimeras were mixed with spleen cells from (B6×CBA)F_1(b.m.) → B10.A(5R)(irr) female chimeras and transferred into irradiated (B6×CBA)F_1 female mice. The adoptive hosts were primed with irradiated (B6×CBA)F_1 male cells and their spleen cells boosted *in vitro* with F_1 male cells 10 to 14 days later. Cytotoxic T cells specific for H-Y antigen in association with *H-2*^{k}-bearing targets were generated from these mixed cell populations, whereas either population alone stimulated under identical conditions generated no H-Y-specific cytotoxic T cells. The magnitude of the cytotoxic response by the mixed chimeric population was lower than that of normal F_1 controls, but was clearly significant. This cellular complementation by two phenotypic nonresponder chimeric spleen cell populations suggested strongly that the two types of *Ir* gene defects responsible for the nonresponsiveness of the chimeras were expressed in separate cell populations and, therefore, that T cell–T cell interactions were likely to be required to generate an H-Y-specific cytotoxic T cell response.

Although similar experimental observations have been made by Matsunaga and Simpson (1978) using (*H-2*^{d}×*H-2*^{k})F_1 animals, the conclusion that an *I* region-restricted cell interaction between T helper cells and precytotoxic T cells is required for generating a cytotoxic response has been seriously challenged. First, several laboratories have demonstrated that it is possible to bypass the need for any form of helper cell activity in the generation of a cytotoxic response by adding lymphokines, such as the mixture of ingredients found in the supernatants of Con A-stimulated spleen cells (reviewed in Nabholz and MacDonald, 1983). This observation has also been made for the cytotoxic response to H-Y (de Waal *et al.*, 1983b). This result suggests that direct cell contact is not necessary between the two cooperating T cells to obtain a cytotoxic response. Second, all attempts to eliminate precytotoxic T cells with anti-Ia antisera and complement, even those sera containing antibodies known to react specifically with T lymphocytes, have failed (Swierkosz *et al.*, 1979). This result suggests that precytotoxic T cells do not bear the

requisite Ia molecules necessary to mediate an *I* region-restricted interaction. Finally, recent studies on the CBA mouse by Müllbacher and Brenan (1980) have demonstrated that this strain can respond to the H-Y antigen if the females are immunized with male cells subcutaneously in the hind foot pads instead of ip or iv, although it took 4–5 weeks for the primed cells to emerge in the spleen instead of the usual 2 weeks seen with B10 mice. These results suggested that the CBA mouse is really a low responder to the H-Y antigen and that, similar to what had already been described for the lysozyme system (see Section VI,C,4) the balance between suppressor and helper cells generated could be manipulated by the route of immunization and thus determine whether responsiveness was detected.

Although these new experiments bring into question the interpretation of the chimera results in terms of a helper T cell–cytotoxic T cell restricted interaction, they do not invalidate the data. The mixing experiment only demonstrated that two cells were required for the cytotoxic response; it did not identify the mechanism by which this occurred. It was the nonresponsiveness of the CBA(b.m.) → (B6×CBA)F_1(irr) chimeras which led to the postulate of a restricted interaction. However, other interpretations of this experimental result are possible. Recall, for example, the B10.M(b.m.) → (B10×B10.M)F_1(irr) chimera experiment in the genetic control of the immune response to (T,G)-A--L (Section VII,C). This chimera was also a nonresponder, even though its T cells matured in a responder environment. It is possible that the interpretation discussed for this entirely *I* region confined *Ir* gene control is also applicable to the low responsiveness of the CBA strain to the H-Y antigen.

Support for this idea comes from the recent experiments of de Waal *et al.* (1983b). They immunized (B6×bm12)F_1 females with F_1 male spleen cells and examined the influence of the *I* region-encoded *Ir* gene on antigen presentation of the H-Y antigen. In contrast to the usual result, in which nonresponder presenting cells fail to stimulate a response, the nonresponder bm12 spleen cells stimulated as potent a cytotoxic response as responder B6 spleen cells. This result was not altered by rigorous depletion of adherent cells from the responder T cell population by passage over Sephadex G-10. This argued against processing of the H-Y antigen by responder F_1 macrophages and subsequent presentation by them to the F_1 T cells as an explanation for the results. Instead, the results suggest that once the "effector" helper T cell is primed, A_β^{bm12}:A_α^b-expressing antigen-presenting cells can stimulate these cells with H-Y. Because the bm12 parental strain cannot generate such T cells, the Ir defect would appear to lie in the generation of help and not in the interaction between the "effector" helper T cell and the precytotoxic T cell. The mechanism by which the generation of help fails to occur has not yet been elucidated. It could involve an *I* region-restricted "inducer" T cell–"effector"

T cell interaction as described in Section VII,C for the DTH response to (T,G)-A--L, or it might involve an *I* region-restricted helper cell-suppressor cell interaction as described in Section VI,C,4,e for the proliferative response to LDH_B.

But no matter what model is invoked, the conclusion still stands that *I* region-restricted help is essential for a cytotoxic response to occur. In this regard, the H-Y system serves as a model of an epistatic effect in which an *I* region-encoded gene prevents the detection of a cytotoxic T cell response specific for an antigen in association with an MHC-encoded class I molecule. Other examples will surely be found when they are looked for more closely.

2. *Immunodominance*

a. The Influence of K^k on the Cytotoxic Response to Antigens Recognized in Association with D^b. The second type of epistatic effect, referred to in the literature as immunodominance, involves the influence of a particular allele at the *K* or *D/L*[5] locus on the cytotoxic response to an antigen in association with an allelic product of another MHC class I-encoding locus, e.g., *D/L* is affected by *K* or *K* is affected by D/L. In the best studied example in the literature, the presence of the K^k allele substantially inhibited the cytotoxic response to vaccinia virus in association with D^b (Zinkernagel *et al.*, 1978; Doherty *et al.*, 1978). Similar observations were made for trinitrophenylated spleen cells (Shearer *et al.*, 1979). This inhibition by K^k was allele specific, because the presence of K^b, K^q, or K^s allowed a strong response to vaccinia virus in association with D^b. For example, B10.A(2R) mice ($K^kI^{k/d}D^b$) did not generate many cytotoxic T cells specific for vaccinia virus in association with D^b (low responders), whereas B10.BYR mice ($K^qI^{k/d}D^b$) did (high responders). The difference between these two recombinant strains located the *Ir* gene to the *K* region, or possibly centromeric to this region, since studies with K^k mutants have not yet been carried out.

The locus and allele controlling the response affected by K^k was unique. First of all, K^k itself was not a low responder allele for vaccinia virus. In a B10.A(2R) mouse (K^kD^b), a strong cytotoxic response was observed to vaccinia virus, and it was all directed against the virus in association with K^k. Second, not all responder *K* alleles had an immunodominant effect. For example, B10 mice responded to vaccinia virus by generating cytotoxic T cells specific for the virus in association with K^b, but they also generated cytotoxic T cells specific for the virus in association with D^b. Third, not all responses to the

[5] At the time many of the experiments to be described were performed, no clear distinction was made at the molecular level between the *D* and *L* loci. Thus, the correct designation for a particular *Ir* gene, *D* or *L*, is not always clear. For the sake of simplicity, except for this sentence, I will use the authors' published notation of only *D* for *Ir* genes in this region; but the reader should keep in mind that in any given case the *Ir* gene could be either *D* or *L*.

antigen were suppressed by the presence of K^k or the generation of a response against the virus in association with K^k. In a (B10×B10.BR)F_1, K^k exists along with K^b and D^b. Immunization with vaccinia virus led to a cytotoxic response to the virus in association with K^b as well as with K^k. Only the response to virus in association with D^b was prevented.

Immunodominance was also antigen specific. K^k did not suppress the response to all antigens in association with D^b. In fact, for the H-Y antigen, Simpson and Gordon (1977) showed that the situation was reversed. Spleen cells from (A/J×B10)F_1 females immunized with (A/J×B10)F_1 male cells were cytotoxic for B10 (K^bD^b) male targets and not A/J (K^kD^d) male targets. Because K^b is a low responder allele for the H-Y antigen, the results suggested that the presence of the D^b allele inhibited the cytotoxic response to the H-Y antigen in association with K^k. However, H-2^k-bearing strains still have the upper hand, as the presence of D^k, e.g., in (CBA×B10)F_1, inhibited the H-Y response in association with both D^b and K^k.

The key difference between the immunodominance phenomenon and a classical cytotoxic *Ir* gene effect is the absence of an intrinsic defect in the MHC allele involved in the response to the antigen. Thus, B10 mice (K^bD^b) immunized with vaccinia virus generated a cytotoxic response to this antigen in association with D^b. Only when the K^k allele was present, e.g., B10.A(2R) (K^kD^b) was this response markedly reduced or eliminated. This was most dramatically demonstrated in F_1 crosses. For example (C3H×B6)F_1 or (B10×B10.BR)F_1 mice, both crosses between K^bD^b- and K^kD^k-bearing strains, failed to mount a strong cytotoxic response against vaccinia virus in association with D^b. Similar results were obtained with F_1s involving the B10.A(2R) (K^kD^b) parental strain. Thus, introduction of the K^k allele on the opposite chromosome eliminated the response to the antigen in association with the D^b gene product. These results show that this type of epistatic effect displays a dominant phenotype in F_1 crosses. This is in contrast to classical cytotoxic *Ir* genes, which behave in a codominant fashion.

The effect of K^k was not on the development of T cells specific for vaccinia virus in association with D^b. These T cells exist in an (H-2^b×H-2^k)F_1 animal. This was demonstrated by transferring mature F_1 T cells into lethally irradiated K^bD^b- or K^kD^b-bearing mice and priming them with vaccinia virus. The nonresponder F_1 T cells were capable of mounting a cytotoxic T cell response to vaccinia virus in association with D^b when transferred into K^bD^b-bearing hosts (e.g., B10) but not when transferred into K^kD^b-bearing hosts [e.g., B10.A(2R) or B10.A(4R)]. Thus, the T cells were present, but if the K^k allele was also present in the priming environment, then the cytotoxic response to vaccinia virus in association with D^b was somehow suppressed. This result suggested that the response to vaccinia virus in association with K^k might play an important role in modulating the response to vaccinia virus in association with D^b.

This hypothesis was tested in two ways. First, Zinkernagel *et al.* (1978) showed with radiation-induced bone marrow chimeras that the cytotoxic T cells must be capable of recognizing K^k as a restriction element. $(H\text{-}2^b \times H\text{-}2^k)F_1$ bone marrow was allowed to develop in either B6 ($K^bI^bD^b$) or B10.A(4R) ($K^kI^{k/b}D^b$) irradiated hosts. The F_1(b.m.) → B6(irr) chimeras responded to vaccinia virus in association with D^b, whereas the F_1(b.m.) → B10.A(4R)(irr) chimeras did not. Thus, having the K^k gene product expressed in the priming environment was not sufficient to suppress the response. The T cells also had to be capable of recognizing it. Second, Bennink and Doherty (1979) showed that the repertoire of a K^k-bearing strain was essential for observing the suppression. They depleted B6 T cells of alloreactive cells to K^k and A^k_β:A^k_α by filtration through a B10.A(4R) mouse. This technique involves iv injection and thoracic duct drainage 1–2 days later (see R. Schwartz, 1984, for the theory behind this technique). Such negatively selected B6 ($K^bI^bD^b$) T cells (designated $B6_{-4R}$), when primed in an irradiated B10.A(4R) ($K^kI^{k/b}D^b$) mouse, were capable of generating a cytotoxic response to vaccinia virus in association with K^k. The mechanism by which this occurs is controversial (see R. Schwartz, 1984, for a review), but is felt to involve cross-reactivity or degeneracy of the B6 repertoire on K^k. Thus, the T cells were fundamentally different from the ones in a K^k-bearing animal that responded to vaccinia virus in association with K^k. Interestingly, the negatively selected B6 T cells also responded to vaccinia virus in association with D^b. Thus, the mere response to vaccinia virus in association with K^k was not sufficient to inhibit the response to vaccinia virus in association with D^b. Rather it would appear that only the repertoire generated in a K^k-bearing mouse would do this. One caveat on this interpretation that should be pointed out is the fact that all the chimera and acute depletion experiments were done with B10.A(4R) mice as the K^k expressor. Since these mice also express A^k_β:A^k_α, it is impossible to tell whether the effects observed were solely due to K^k expression or also involved *I* region effects.

b. Biological Mechanism(s). Overall, the results suggest that the influence of the K^k allele on the response to vaccinia virus in association with D^b is a true epistatic effect. In the absence of K^k, T cells specific for vaccinia virus in association with D^b were shown to exist and to function normally. In the presence of the K^k gene product in the priming environment and a T cell repertoire that developed in the presence of the K^k gene product, the response to vaccinia virus in association with D^b was selectively inhibited. The mechanism by which this occurs is not clear (see Longo *et al.*, 1981, for an extensive discussion of all the mechanisms that have been proposed to explain this phenomenon). The most likely explanation is a competition between the T cells specific for vaccinia virus in association with K^k and those T cells specific for the virus in association with D^b. For example, if the former

were generated from precursors with higher affinity or of greater number, then they might out-compete precursors of the latter for limited amounts of IL-2 produced by helper T cells. Alternatively, the former clones might expand more quickly, activate a nonspecific inhibitory mechansim (e.g., killing off the stimulator cells as described by Pang and Blanden, 1976) and thus limit the expansion of the latter clones (see Grossman, 1984, for a detailed discussion of this balance of growth model).

Müllbacher *et al.* (1983) have tested the nonspecific inhibition model by comparing the response of double bone marrow chimeras to that of control F_1 pseudochimeras. $H\text{-}2^k$(b.m.) + $H\text{-}2^b$(b.m.) → ($H\text{-}2^k \times H\text{-}2^b$)$F_1$(irr) chimeras gave the same weak cytotoxic response to vaccinia virus in association with D^b as ($H\text{-}2^k \times H\text{-}2^b$)$F_1$(b.m.)→ ($H\text{-}2^k \times H\text{-}2^b$)$F_1$(irr) pseudochimeras. Therefore, it seems unlikely that the rapid generation of cytotoxic T cells specific for vaccinia virus in association with K^k is preventing the response to vaccinia virus in association with D^b by eliminating the stimulator cells bearing D^b, because in the double bone marrow chimeras the K^k and D^b molecules were expressed on different cells (unless of course the stimulator was a radio-resistant F_1 host cell).

Another problem with these competitive inhibition type of models is that they do not simply explain why the suppression is specific for vaccinia virus in association with D^b. There appears to be no effect, for example, on cytotoxic T cells specific for vaccinia virus in association with K^b. Although this might be explained by postulating that the K^b-specific precursors are also present in large enough numbers or are of high enough affinity to compete for limited factors (but not so great that they inhibit the T cells specific for vaccinia virus in association with D^b), Müllbacher *et al.* (1983) have suggested another possibility. They neonatally tolerized B10 mice to K^k and A^k_β·A^k_α by injecting neonatal animals with [B10×B10.A(4R)]F_1 cells. At 6 weeks of age these mice were challenged with vaccinia virus and shown to mount a cytotoxic response to the virus in association with K^b but not with D^b. In contrast, neonatal tolerance of B10 mice to K^q and I^k using (B10×BYR)F_1 spleen cells did not result in suppression of the response to vaccinia virus in association with D^b. In neither case was a response to vaccinia virus in association with K^k detected. The suppression induced by [B10×B10.A(4R)]F_1 cells was antigen specific in that the cytotoxic response to LCM virus in association with D^b was not suppressed. Thus, merely tolerizing to K^k specifically diminished the cytotoxic response to vaccinia virus in association with D^b, even in the absence of a response to vaccinia virus in association with K^k. Based on the observation in a few isolated cases (individual mice) of cross-reactive cytotoxic activity on uninfected K^k targets by T cells specific for vaccinia virus in association with D^b (demonstrated only by cold target inhibition), Müllbacher *et al.* (1983) argued that some

form of active tolerance mechanism (e.g., suppressor T cells specific for an idiotype on the cytotoxic T cells recognizing both K^k and vaccinia virus in association with D^b) accounted for the results. However, this explanation cannot account for the (H-$2^k \times H$-2^b)(b.m.) → H-2^b(irr) chimera and $B6_{-4R}$ acute depletion experiments discussed above, in which the animals were tolerant to K^k and K^k was present in the priming environment, yet they made a strong response to vaccinia virus in association with D^b. Thus, at the present time, no agreed upon explanation for immunodominance has been put forth. It is possible that distinct mechanisms are operating in the neonatal tolerance model as compared to the other tolerance models.

c. I Region Analogies. In the case of *I* region-encoded *Ir* genes, the closest analogy to this type of epistatic mechanism is the suppressive effects described in Section VI,C,4. In this case, suppressor T cells specific for antigens such as LDH_B in association with E^k_β:E^k_α inhibited the proliferative response of T cells specific for the same antigen in association with A^k_β:A^k_α. When the E^k_β:E^k_α Ia molecule was not expressed, as in the B10.A(4R) mouse, then a proliferative response to LDH_B in association with A^k_β:A^k_α was observed. It was also allele specific in the sense that only E^k_β:E^k_α elicited such suppressor cells. However, lack of extensive studies with F_1 mice, or any studies with chimeric animals preclude any further comparisons between this suppression model and the immunodominance observed in cytotoxic T cell systems.

Another analogy, although somewhat convoluted, can be made with certain phenomena in *I* region-controlled responses, if the idea of competition between T cell clones is a valid explanation of the phenomenon of immunodominance. It has been observed in a number of *I* region-controlled systems, that the response to one antigenic determinant on a multideterminant antigen precludes the response to another determinant on the same molecule, although neither determinant is eliciting detectable suppressor T cells. For example, when B10 mice are immunized with either (T,G)-A--L or (Phe,G)-A--L, T cells from the peritoneal cavity will proliferate *in vitro* when challenged with either (T,G)-A--L or (Phe,G)-A--L (R. Schwartz *et al.*, 1977). This indicates that the two antigens share a cross-reactive determinant. However, when B10.D2 mice are immunized with these two antigens, they manifest a phenomenon known as a one-way cross-reaction. Immunization with (T,G)-A--L elicited T cell clones that could be stimulated either by (T,G)-A--L or (Phe,G)-A--L in association with A^d_β:A^d_α. In contrast, when immunized with (Phe,G)-A--L, the B10.D2 T cell clones elicited only reacted with (Phe,G)-A--L. These results suggest that the B10.D2 mouse has two sets of T cells, one specific for a unique determinant on (Phe,G)-A--L, the other which can recognize a determinant shared by (T,G)-A--L and (Phe,G)-A--L. Immunization with (T,G)-A--L only elicited the cross-reactive

population because (T,G)-A--L lacks the unique (Phe,G)-A--L determinant. Immunization with (Phe,G)-A--L gave the unexpected result. Only the population specific for the unique (Phe,G)-A--L determinant was elicited, even though (Phe,G)-A--L bears the cross-reactive determinant and the B10.D2 T cell population has T cell clones that can recognize it. In other words, the response to the unique determinant on (Phe,G)-A--L in association with A^d_β:A^d_α somehow suppressed the response to the cross-reactive determinant in association with A^d_β:A^d_α. This effect has been referred to as the immunodominance of one determinant over another (R. Schwartz *et al.*, 1977). The biological explanation for this phenomenon is unknown.

At first glance the observations on the proliferative response to copolymers seems unrelated to the immunodominance phenomenon of cytotoxic T cells. This is because the former involves the relationship between different antigenic determinants in the presence of a common *I*-region gene product, while the latter involves the relationship between different class I molecules from separate loci in the presence of a common antigen. However, the patterns in the two relationships are identical. For example, immunizing B10.D2 mice with (T,G)-A--L to elicit the cross-reactive proliferative T cell clones is formally analogous to immunizing F_1 cytotoxic T cells against vaccinia virus in association with D^b by priming the cells in a B10 mouse that lacks K^k. In both cases the response that leads to the suppression is not allowed to occur. Thus, it is not unreasonable to speculate that the immunodominance in the two systems might have the same underlying mechanism if one examines the two systems from the point of view of recognition units, i.e., foreign antigen plus MHC-encoded molecule. In one case the units change by varying the foreign antigen, in the other case by varying the class I molecule. The effect is the same: the activation of a set of clones that inhibit the response of other T cells in some as yet unknown manner.

3. *Allogeneic Cross-Reactive Lysis by Cytotoxic T Cells Specific for TNP-Modified Self*

Given the biologic complexity of the cytotoxic T cell response, it would not be surprising to find some cases of *Ir* gene control that involved several different mechanisms. One example of this appears to be in the cytolytic T cell response to trinitrophenyl (TNP)-modified syngeneic spleen cells (TNP-self). Billings *et al.* (1978a) discovered a specificity difference between mice bearing the *H*-2^k haplotype and all other strains. Whereas Burakoff *et al.* (1976) had shown that cytotoxic T cells elicited by spleen cells derivatized with 10 m*M* TNP demonstrated cross-reactive lysis on target cells bearing allogeneic K and D molecules, Billings *et al.* (1978a) found that mice bearing the *H*-2^k haplotype did not do this. The Ir effect was not a failure to generate cytotoxic T cells specific for TNP in association with *H*-2^k-encoded class I

molecules (K^k), but rather the failure to generate a set of cytotoxic T cells with a particular cross-reactive specificity.

Responsiveness appeared to be dominant. For example, (B10×B10.BR)F_1 spleen cells stimulated with either TNP-derivatized B10 ($K^bI^bD^b$) or B10.BR ($K^kI^kD^k$) spleen cells developed cross-reactive cytotoxic T cells. Thus, even stimulator cells from the low responder strain (B10.BR) could elicit a cross-reactive response. This suggested that there was no problem in antigen presentation as one usually observes for classic *Ir* gene effects (see Section VIII,B,1,c). This conclusion was supported by experiments with P(b.m.) → F_1(irr) chimeras (Billings *et al.*, 1978b), in which spleen cells from C3H ($K^kI^kD^k$)(b.m.) → (B6×C3H)F_1(irr) mice generated cross-reactive cytotoxic T cells. The TNP-derivatized presenting cells were of low responder C3H type, but the T cells had matured in a responder F_1. Thus, the Ir defect appeared to be solely in the T cell repertoire. Note, however, that this phenotype is distinct from that of the *I* region-encoded *Ir* genes controlling the cytotoxic response to the H-Y antigen. In the present example, the chimeric low responder T cells were able to generate a cross-reactive response; in the H-Y case, they did not respond (see Section VIII,C,1,b for comparison).

Initial genetic mapping studies (Billings *et al.*, 1978a) localized the critical MHC-encoded molecules to the *K* region and/or *I-A* subregion. Thus, B10.A(4R) mice (K^k, $A^k_\beta A^k_\alpha$, D^b) did not generate cross-reactive cytotoxic T cells, but A.TL mice (K^s, $A^k_\beta A^k_\alpha$, D^d) and B10.AQR mice (K^q, $A^k_\beta A^k_\alpha$, D^d) did. Surprisingly, mutation of the K^k gene, as found in the M523 mouse (K^{ka}, I^k, D^k) (Blandova *et al.*, 1975), did not alter the Ir phenotype. However, it also did not alter the cytotoxic T cell specificity, since M523 (K^{ka}) cytotoxic T cells specific for TNP-self would lyse TNP-modified CBA (K^k) target cells and vice versa.

To dissect the roles of the class I and class II genes in this response, Burakoff *et al.* (1980) bred F_1 hybrids that were heterozygous only at the *K* region. (A×A.TL)F_1 mice are (K^k×K^s) heterozygotes, but homozygous in the *I-A* subregion (I-A^k). When primed *in vitro* with TNP-derivatized A.TL (K^s) cells, the F_1 spleen cells generated cross-reactive cytotoxic T cells. In contrast, when primed with TNP-derivatized A (K^k) cells, they did not generate cross-reactive cytotoxic T cells. Thus, heterozygosity only at the *K* locus revealed that K^k was a classical-type, cytotoxic *Ir* gene. T cells specific for TNP-derivatized K^k, which also showed cross-reactive lysis on allogeneic K or D molecules, were not stimulated or did not exist in the (A×A.TL)F_1.

To examine the role of the *I* region, the response of the (A×A.TH)F_1 mouse was compared to that of the (A×A.TL)F_1 mouse. The (A×A.TH)F_1 introduces heterozygosity at the *I-A* subregion (A^k_β:A^k_α×A^s_β:A^s_α). This strain

showed cross-reactive lysis on allogeneic targets when immunized with TNP-derivatized A spleen cells (K^kI^k). Because the (A×A.TL)F_1 strain did not generate cross-reactive T cells, the results suggested that the responder allele of an *I*-region-encoded *Ir* gene was capable of overcoming the non-responsiveness imparted by the classical-type cytotoxic *Ir* gene. This interpretation assumes that the K^s allele was not necessary for the response.

The mechanism by which K^k and A^s_β:A^s_αcould interact in this response is not known. One possible way would be to postulate an immunodominance-like effect in the cytotoxic response. In this scenario two subpopulations of T cells would exist in *H*-2^k-bearing mice that are both specific for TNP-derivatized K^k, but only one of which would show cross-reactive lysis on allogeneic targets. In addition, A^k_β:A^k_α would be a low responder Ia molecule leading to the generation of only a small amount of T cell help during the response. If the non-cross-reactive T cells had a higher affinity for the antigen or were present in greater number, then they could effectively compete for the limited amount of IL-2 generated by the helper T cells and dominate the response. Since introduction in the F_1 of K^s, which by itself leads to the generation of cross-reactive cytotoxic T cells, did not overcome the defect, the competitive edge of non-cross-reactive, TNP-K^k-restricted T cells would also have to be dominant over T cells specific for TNP-derivatized self molecules in association with K^s. This is different from the viral immunodominance model discussed in Section VIII,C,2,a. Finally, introduction of $A^s_\beta A^s_\alpha$ would change the balance in favor of the cross reactive T cell clones. How this would come about is the greatest puzzle of all. If one postulated that this occurred by creating more helper T cells, which would generate more IL-2 and allow the cross-reactive clones to expand, then this could only occur through a positive selection mechanism in the thymus (see Section VI,C,3). This is because the helper T cells must be specific for TNP-derivatized self molecules in association with $A^k_\beta A^k_\alpha$. If so, it would be the first demonstration of such an effect at the helper T cell level. Alternatively, the increased help could derive from the processing of TNP-A^k_β:A^k_α by F_1 macrophages and re-presentation in the context of A^s_β:A^s_α.

But whatever the mechanism, it is clear that the interaction between an *I* region-restricted set of helper T cells and a *K* region-restricted set of cytotoxic T cells determined the outcome of the immune response. Particular alleles at each genetic locus were required to generate the low responder phenotype. In this sense the allogeneic cross-reactive lysis by cytotoxic T cells specific for TNP-modified self represents a good example of an epistatic effect. Another potential example, which has not been as well studied, is the *I* region control of the graft rejection of skin from mice differing at the H-4 minor histocompatibility locus (Wettstein and Haughton, 1977).

IX. Conclusion

The study of *Ir* genes represents a microcosm of the development of cellular immunology. The initial discovery of a biological phenomenon, the genetic inheritance of responsiveness and nonresponsiveness to synthetic polypeptides by outbred and inbred strains of animals, led eventually to a full genetic and cellular characterization of the critical first step in the initiation of an immune response. This step is the recognition by T lymphocytes of foreign antigens in association with gene products of the major histocompatibility complex. We now are in the final phases of our understanding of this event as molecular biologists clone the critical genes involved and biochemists characterize the interactions between the various proteins. This movement from biological phenomenon, to cellular characterization, to molecular understanding is really the progress of all biological science. The purpose of this review was to try and capsulize this natural progression in one area of immunology so that scientists entering into the field at the molecular level could have an appreciation for the roots from which their problem developed.

Looking back, it is clear that our understanding of T lymphocyte development and recognition has improved enormously over the past 25 years. Yet, a full understanding of *Ir* gene effects, the ways in which MHC gene products influence the immune response in an antigen- (or determinant-) specific way, has still not fully crystallized. I have discussed in detail the places at which this might occur: during the evolution of the T cell *V* region repertoire, during T cell development (either through positive selection of the repertoire or through negative selection during tolerance induction), during antigen processing and presentation (if the antigen and the MHC-encoded molecule physically interact), or during the immune response itself when cellular interactions such as suppression or the quantity of T cell help (in a cytotoxic response) determine the final outcome of the response. Some experimental evidence exists to support most of these mechanisms. However, the quantitative impact that each has on the immune response is the critical unknown. Although some investigators in the field feel strongly that one mechanism or another plays the key role, my own feeling is that most of them will contribute, although to varying degrees depending on the foreign antigen being recognized.

There are also a number of other questions in the field which still remain unanswered. For example, how do *Ir* genes influence the B cell response in a determinant specific way? Recall from Section III,A that certain B cell responses lack the formation of antibodies specific for the same determinant that T cells fail to recognize. How this occurs is difficult to imagine, since B cell receptors recognize free antigen, whereas helper T cells recognize the

determinant in association with class II molecules. Any repertoire deletion model would have to be inordinately complex. Recently, however, Berzofsky (1983) has proposed an antigen presentation model that could explain the observation. B cells recognize antigenic determinants on the intact molecule via their immunoglobulin receptors, in contrast to macrophages which take up antigen without the use of a specific receptor. If T cells help B cells by first recognizing antigen fragments on the surface of the B cell in association with class II molecules, then the nature and quantity of the fragments processed by B cells become critical for the activation of these cells. Berzofsky proposed that the immunoglobulin receptor altered the processing by selectively protecting the determinants bound to the antibody. The net result of this protection would be to have those B cells that are specific for the same (or a nearby) determinant recognized by T cells express more of this fragment on their surface. This would increase the chances that these particular B cells would be stimulated in a responder strain. Conversely, B cells with specificity for determinants on regions of the molecule not recognized by T cells (nonresponder status) would protect and express fragments that could not be recognized by the T cell population. Therefore, these B cells would not be stimulated. In this manner the specificity of the B cell response could mimic the specificity of the T cell response. This hypothesis remains to be experimentally tested.

Another area in which many questions still remain is the role of class II molecules in T cell–T cell interactions. As discussed in Section VII, even the fundamental question of whether these molecules are expressed in murine T cells remains unanswered. It is difficult to imagine that the mouse could be different from other mammals, such as guinea pigs and humans, which have been shown to possess T cells that express class II molecules when activated. On the other hand, the failure to detect mRNAs encoding these molecules in molecular biology experiments that are quite sensitive and specific imparts a strong degree of doubt as to their expression in these cells. More than likely the problem lies in our failure to fully understand the biology of T cell regulatory interactions. This is a complex area of cellular immunology (see D. Green *et al.*, 1983, for a review) and possibly several intermediate cell types are still missing from our equations. Hopefully, when we finally do understand the cell biology and biochemistry, problems such as the nature of the *I-C* subregion will also be solved.

Finally, there is one important question remaining that cellular and molecular immunologists may never be able to definitively answer. This question is why the immune system has to tolerate immune response defects at all? Clearly, from a practical point of view, if one were to design the best of all possible immune systems, one would want it to recognize all possible foreign substances. The inability of certain individuals to recognize particu-

lar foreign substances suggests that compromises had to be made in order to create a viable system. One of these was undoubtably the need not to respond to self molecules, an event that could have disastrous consequences for the organism. But why different individuals would have different defects is not obvious from a simple self tolerance model. The answer that has been put forth by a number of different investigators (Doherty and Zinkernagel, 1975; Langman, 1978; R. Schwartz, 1978; Klein and Figueroa, 1981) is a teleological argument involving population genetics. If the need for self–nonself discrimination precludes recognition and response to all possible chemical shapes, then a foreign organism (infectious agent) would only need to coat itself with chemical shapes that mimicked some of those found in the host it wished to invade, in order for it to escape surveillance by that host's immune system. On the contrary, if each individual in the species was unique, and possessed an immune system with different recognition potential, then the infectious agent could not adapt to all the possible responses. As a consequence, the species would be protected against any given infectious agent, although particular individuals in the species would be vulnerable. Because different infectious agents are likely to have different chemical structures on their surface, different individuals in the population would be more or less susceptible to different diseases. Therefore, a mixture of selection forces (different diseases) would lead to a stable population with a large degree of polymorphism in which any given allelic form would be present at a low frequency (Lewontin *et al.*, 1978). This description, of course, fits the nature of the genes in the MHC. Thus, it is possible that recognition by T cells of antigen in association with the polymorphic gene products of the MHC represents a mechanism by which the species circumvents the problem created by self–nonself discrimination. In this sense the unique nature of *Ir* gene defects derives from the requirement to distribute the defensive burden among different members of the species.

ACKNOWLEDGMENTS

I would like to thank Drs. Ron Germain, Jon Ashwell, Helen Quill, Misha Sitkovsky, and Barbara Fox for critically reviewing the manuscript and Ms. Shirley Starnes for her excellent editorial assistance. I would also like to thank my wife, Joan Schwartz, for her patience and support during the many months required to produce this review.

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ADVANCES IN IMMUNOLOGY, VOL. 38

The Molecular Genetics of Components of Complement

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I. Introduction

Investigation of the phenotypic genetics of complement components (Hobart, 1984) and of inherited deficiencies of the components (Lachmann, 1984) has established linkage of many of the complement component genes with each other and with genes coding for other proteins. Most striking perhaps was the mapping of the genes coding for C2, Factor B, and C4 to the major histocompatibility complex (MHC) in man (HLA) on chromosome 6 between HLA-D and HLA-B reported in a series of papers summarized by Weitkamp and Lamm (1982). In mice, these complement genes are also in the MHC, H2, between I and D. Estimates of map distances between the loci are given in Fig. 1 but, as discussed later, their accuracy is uncertain. The gene coding for the Ss protein was first mapped to H2 (Shreffler and Owen, 1963) and later identified as C4 (Meo *et al.*, 1975; Lachmann *et al.*, 1975; Curman *et al.*, 1975; Carroll and Capra, 1978). Subsequently Factor B

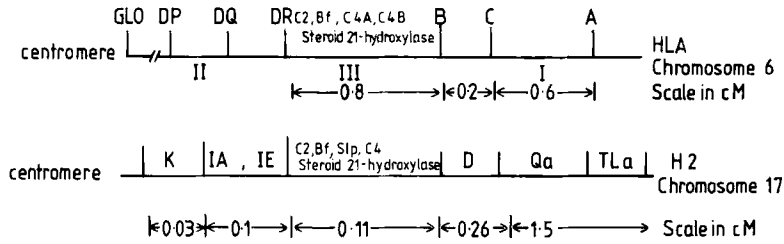

FIG. 1. Alignment of loci in the HLA and H2 complexes with estimates of distances between loci in cM. Data for H2 from Chaplin *et al.* (1984) and for HLA from Barnstable *et al.* (1979).

(Roos and Demant, 1982) and C2 (Gorman *et al.*, 1980) were also linked to the S region. C6 and C7 are closely linked (Hobart *et al.*, 1975, 1978) but have not yet been allocated to a chromosome. C3, C4 and C5 though structurally homologous are not linked. The C5 gene has not been placed, but in man the C3 gene is on chromosome 19 (Whitehead *et al.*, 1982) while C4 is in the HLA locus. In mouse, the C3 gene is on chromosome 17 but it is 10–12 cM telomeric of C4 in the H2 locus (Da Silva *et al.*, 1978; Natsuume-Sakai *et al.*, 1978).

Recently, a very interesting linkage has been shown in man between the genes for C4b-binding protein (C4bp), Factor H and the C3 receptor CR1 (Rodrigues de Cordoba *et al.*, 1984, 1985a) but it is not yet known on which chromosome. The relationship between the genes coding for these proteins in the mouse is less clear. Polymorphism of Factor H has enabled the gene to be mapped to chromosome 2 (Natsuume-Sakai *et al.*, 1985) but the gene for C4bp had been placed previously on chromosome 17 in the H-2D-Qa section (Kaido *et al.*, 1981; Takahashi *et al.*, 1984b). However, Rodriguez de Cordoba and colleagues (1985b) were unable to confirm the polymorphism of C4bp on which the mapping was based. It is uncertain therefore whether C4bp and Factor H genes are or are not linked in the mouse as has been found in man. As discussed later C4bp, Factor H, and CR1, though functionally distinct, do have related activities.

Linkage might be expected between the genes coding for C1r and C1s as these serine proteases are very similar in general structure (Villiers *et al.*, 1985) and in such comparable amino acid sequence as is available. However, no data on the genetics of C1r and C1s appears to be available so far.

Phenotypic genetics have also established the exceptionally high polymorphism of C4 (Mauff *et al.*, 1983) which is comparable with that found in the Class I and Class II MHC antigens. Further studies are likely to relate the genes coding for the complement components to each other and with somatic cell genetics to place them on the different chromosomes. More precise mapping, however, will be dependent on the cloning of individual

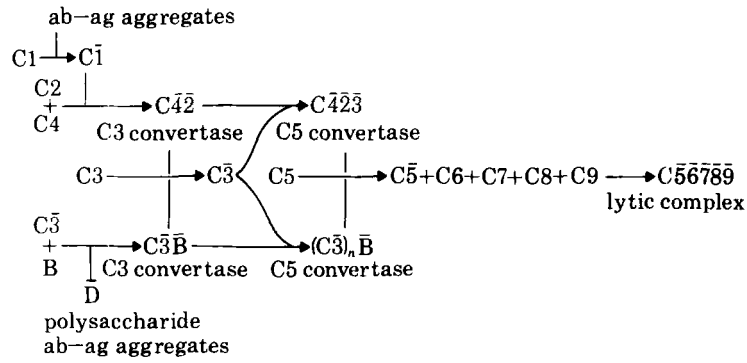

FIG. 2. Activation pathways of complement. The upper section shows the classical pathway activated *in vivo* primarily by antibody–antigen aggregates. The first component C1 is formed from 3 subunits C1q, C1r$_2$, and C1s$_2$ associated in the presence of Ca^{2+}. Binding of C1 through C1q to the Fc section of the Ig leads to the conversion of C1r and C1s to active proteases. $\overline{C1s}$ then activates C4, which binds covalently to the aggregates, and C2 which associates noncovalently with bound C4 to form the C3 convertase $C\overline{4\bar{2}}$. Activated $C\bar{3}$ binds covalently to form C5 convertase $C\overline{4\bar{2}\bar{3}}$ and the activated $C\bar{5}$ associates spontaneously with the late components C6, C7, C8, and C9 to form the lytic complex. The lower section shows the alternative pathway initiated either by antibody–antigen aggregates or other substances such as high-molecular-weight polysaccharides found in microorganisms. Activated $C\bar{3}$ associates with factor B which is then activated by $\bar{D}$ a protease present in plasma as an active enzyme. $C\overline{3B}$ which is formed is a C3 convertase and activated $C\bar{3}$ binds covalently with this enzyme and forms $(C\bar{3})_n\overline{B}$ which is a C5 convertase. The activated $C\bar{5}$ forms a lytic complex as when activated in the classical pathway. Not shown are a series of control proteins which limit the activation cascade.

genes and chromosome walking but new techniques such as orthogonal field electrophoresis (Schwartz and Cantor, 1984; Van der Ploeg *et al.*, 1984; Carle and Olsen, 1984) and directional cloning (Collins and Weissman, 1984) should increase the rate of progress.

The functional relationships of the different components of complement in the activation scheme (reviewed in Reid and Porter, 1981; Müller-Eberhard, 1983) are shown in Fig. 2.

There are two pathways of activation, initiated in different ways but probably most commonly *in vivo* by the formation of antibody–antigen aggregates in the blood in the classical pathway and also by the presence of high-molecular-weight polysaccharides in the alternative pathway. Each step involves the conversion of a proteolytic zymogen into an active enzyme until after the activation of C5 which then forms a lytic complex common to both pathways, in association with C6, C7, C8, and C9. Special features of the system are that each of the proteases are complexes of 2 or 3 proteins and they are bound noncovalently or covalently to the activating substances. The C3 and C5 convertases of the two pathways have equivalent specificities but

are formed from different though structurally and functionally related proteins. Thus, several proteins can be paired such as C2 and Factor B which carry the proteolytic activity of both the C3 and C5 convertases in the classical and alternative pathways, respectively. C3 and C4 are also similar in structure and function, but C3 has an additional role in that when associated with either of the C3 convertases it changes the specificity to that of a C5 convertase. The C5 protein though quite distinct in function is structurally related to C3 and C4.

There are several control mechanisms in the complement activation scheme which limit its activity and prevent damage being caused to the animal's own tissues. The binding of the complex proteases to the activating particles localizes the activity and each has only a short half life due to the dissociation of C2 or Factor B from the complex. $C\bar{1}$ inhibitor displaces and inactivates the proteases $C\bar{1}r$ and $C\bar{1}s$ in the $C\bar{1}$ complex (Ziccardi and Cooper, 1979; Sim *et al.*, 1979), Factor I hydrolyzes peptide bonds in C3b with cofactor H (Pangburn *et al.*, 1977) and in C4b with cofactor C4bp (Scharfstein *et al.*, 1978) in both cases destroying their activity. Conversely properdin stabilizes the C3 and C5 convertases of the alternative pathway. Protein S, in the plasma, combines with and inactivates the lytic complex in the blood (Podack and Müller-Eberhard, 1978). The combined effect of these control proteins is to restrict the activation of complement to the immediate environment of the activating substances. This is reinforced by the presence in the blood of high concentrations of other proteolytic inhibitors which serve to control activation systems, such as blood clotting and lysis, which are also dependent on the conversion of zymogens to active proteases.

Another feature of the system is its function in the removal of antibody–antigen complexes, the failure of which leads to immune complex disease. The covalent binding of C4 and more importantly, C3, to the aggregates causes partial dissolution (Czop and Nussenzweig, 1976) and speeds their removal due to the presence of C3b and C4b receptors on the surfaces of red blood cells and phagocytic cells (reviewed by Fearon, 1984).

Thus, there are 3 proteins all of which bind the similar proteins, C3b and C4b. These are Factor H and C4bp, cofactors for the digestion of C3b and C4b, respectively, and the C3/C4 receptor (CR1) which binds both C3b and C4b. Some structural similarity would be expected and the recent finding that these genes are linked supports this view.

While the phenotypic and deficiency studies have provided the basis of the genetics of complement components and will continue to make a major contribution to the mapping of the genes, the detailed relationships and the full extent of polymorphisms and the establishment of the primary structures from derived amino acid sequences are now being provided by the application of recombinant DNA techniques.

This article will summarize the present position with emphasis on the MHC Class III genes for which most data are published but also including such information on the molecular genetics of the other components as is available. Recent reviews of most aspects of the complement system will be found in Müller-Eberhard (1983) and Porter *et al.* (1984).

II. C2 and Factor B

C2 and Factor B are class III gene products of the MHC. Both are single chain glycoproteins of 100,000 and 92,000 M_r, respectively, and upon activation are cleaved at a single peptide bond to yield N-terminal fragments of 30,000 M_r (Ba and C2b) and C-terminal fragments of 60–70,000 M_r (Bb and C2a) (reviewed in Reid and Porter, 1981). Like all glycoproteins coded for by the MHC both demonstrate genetic polymorphism, but to a lesser extent than most other MHC products.

Factor B variants are detected by agarose gel electrophoresis followed by immunofixation (Alper *et al.*, 1972). In man the patterns have been interpreted as representing four allelic forms at a single autosomal locus. The common variants F and S have gene frequencies in Caucasoids of 0.17 and 0.81, respectively, while the less common variants F_1 and S_1 have gene frequencies of 0.006 (Olaisen *et al.*, 1975; Albert *et al.*, 1977), though these figures vary widely in different ethnic populations (Alper, 1981). In addition, up to 14 rare variants have also been described (Mauff *et al.*, 1978; Dykes *et al.*, 1983). The structural differences which define the F and S variants are thought to lie in the Ba fragment while those defining F_1 and S_1 and some of the rare variants are thought to lie in the Bb fragment (Alper *et al.*, 1972; Mauff *et al.*, 1978). Further subdivision of the common variants F and S has been achieved by isoelectric focusing (Teng and Tan, 1982; Geserick *et al.*, 1983; David *et al.*, 1983; Abbal *et al.*, 1985), suggesting that the number of alleles may be higher than previously recognized. No functional difference has so far been detected between the F and S protein variants, but it has been reported that individuals who are F homozygous have a higher concentration of Factor B in their plasma than individuals who are S homozygous (Mauff *et al.*, 1980b; Mortensen and Lamm, 1981).

Genetic variation of Factor B has also been seen in other species including mouse, guinea pig, rhesus monkey, and chimpanzee. Polymorphism in mouse Factor B was first suggested by Rubinstein *et al.* (1977). Further analysis using agarose gel electrophoresis (Roos and Demant, 1982) or isoelectric focusing (Natsuume-Sakai *et al.*, 1983) followed by immunofixation has confirmed and extended this observation and established that mouse Factor B has at least two (Roos and Demant, 1982) and possibly four (Natsuume-Sakai *et al.*, 1983) alleles at a single autosomal locus. Variants of mouse Factor B have also been identified by 2-D peptide mapping (Natsuume-

Sakai *et al.*, 1984) and by differences in activity in a hemolytic assay (Paolucci and Shreffler, 1983). Analysis of the deglycosylated molecules has suggested that the polymorphism is not due to a variation in carbohydrate content (Roos, 1984). In the guinea pig (Bitter-Suermann *et al.*, 1977), rhesus monkey (Ziegler *et al.*, 1975a), and chimpanzee (Raum *et al.*, 1980), as in man, variants have been identified by agarose gel electrophoresis and immunofixation. In contrast to the situation with human Factor B the genetic variation defining the major allotypes in guinea pig and rhesus appears to be carried in the Bb fragment.

Homozygous deficiency of Factor B has not been seen in any animal species suggesting that it may be lethal, and reports of heterozygous deficiency are also rare. However, in two recent studies a heterozygous deficiency of human Factor B was described which was thought to be due to a hemolytically inactive gene product (Mauff *et al.*, 1980a; Suciu-Foca *et al.*, 1980), while in a third the existence of a silent allele was indicated (Tokunaga *et al.*, 1984).

C2 variants are detected by isoelectric focusing followed by hemolytic overlay. In man, one common variant (C2C gene frequency 0.97) and two rare variants have been described: C2A and C2B (Alper, 1976; Hobart and Lachmann, 1976; Meo *et al.*, 1977). Genetic polymorphism of C2 has also been seen in guinea pig (Bitter-Suermann *et al.*, 1981) and chimpanzee (Raum *et al.*, 1980) by the same method, and in mouse by micropeptide mapping (Takahashi *et al.*, 1984a).

In contrast to the situation with Factor B, C2 null alleles in man (Glass *et al.*, 1976; Pariser *et al.*, 1978; Mortensen *et al.*, 1980) and guinea pig (Bitter-Suermann *et al.*, 1981) have been observed. In man, deficiency of C2 is the most prevalent genetic disorder of complement proteins found with a gene frequency of 0.5–1% (Glass *et al.*, 1976), though deficiency of either of the C4 classes is also common as discussed later. Fu *et al.* (1974) first suggested a linkage between certain HLA types and C2 deficiency. Subsequently linkage between C2 structural variants and MHC haplotypes provided evidence that the structural gene was within the HLA region (Meo *et al.*, 1976, 1977; Hobart and Lachmann, 1976; Olaisen *et al.*, 1978). Similarly the Factor B locus was also shown to be HLA linked (Allen, 1974; Olaisen *et al.*, 1975; Hauptmann *et al.*, 1976; Lamm *et al.*, 1976) and comparison of C2 and Factor B allotypes established a close linkage between these genes (Alper, 1976; Raum *et al.*, 1979). Studies of the inheritance of the polymorphic forms of C2, Factor B, and various recombinant MHC haplotypes have placed the structural genes for C2 and Factor B between the HLA-B and HLA-D loci (Olaisen *et al.*, 1981, 1983; Weitkamp and Lamm, 1982; Robson and Lamm, 1984). The genetic variants of Factor B and C2 seen in other species has allowed the genes for these two proteins to be mapped to the H-2 complex of the mouse (Roos and Demant, 1982; Natsuume-Sakai *et al.*, 1983, 1984;

Takahashi *et al.*, 1984a), and to the MHC of the guinea pig (Kronke *et al.*, 1977; Bitter-Suermann *et al.*, 1981) and chimpanzee (Raum *et al.*, 1980), and the Factor B gene to the MHC of the rhesus monkey (Zeigler *et al.*, 1975b). These analyses further confirm the extensive homology of the MHC among higher vertebrate species.

The recognition of genetic deficiencies and polymorphisms of C2 and Factor B has prompted studies of the genetic organization and structure of the loci encoding these two complement proteins. This analysis has mainly been concerned with the human HLA Class III region and with the S region in the H-2 complex of the mouse and has been made possible by the isolation of cDNA clones containing inserts corresponding to C2 and Factor B.

A. Molecular Cloning of Factor B and C2 cDNA

The experimental approach adopted to isolate clones specific for C2 and Factor B has been to synthesize oligonucleotides against a region of the available amino acid sequence such that all possible coding alternatives are present in the mixture. Woods *et al.* (1982) used two 17-base mixtures of 32 and 48 synthetic oligonucleotides, respectively, as hybridization probes, to isolate 19 positive clones from a liver cDNA library with inserts in the range 1–2.3 kb. Sequence analysis of the longest clone pBfA28 showed it to contain coding information for amino acids 63–739 of Factor B including the 3′-untranslated region of the mRNA (Mole *et al.*, 1984). Campbell and Porter (1983) on the other hand used a 14-base mixture of 8 synthetic oligonucleotides as a specific primer of cDNA synthesis and determined part of the nucleotide sequence of the primed product. On the basis of this result a unique 17 base oligonucleotide was synthesized and used as a hybridization probe to isolate two clones from a cDNA fragment library. Subsequently, screening of a full-length cDNA library with Factor B-specific cDNA and genomic probes yielded 11 clones with inserts in the range 1.5–2.3 kb (Morley and Campbell, 1984). The longest clone pFB3b contains coding information for the complete protein molecule, 10 amino acids of a putative leader peptide and the 3′-untranslated region of the mRNA which is 56 bases long and contains the variant polyadenylation signal AUUAAA beginning 20 bases before the poly(A) tract.

The complete amino acid sequence of Factor B has been derived from both protein and DNA sequencing (Christie and Gagnon, 1983; Mole *et al.*, 1984; Morley and Campbell, 1984). The zymogen is composed of 739 amino acids and comparison with the zymogens of other serine proteinases did not reveal any sequence homology over the N-terminal two-thirds of the molecule. However, as shown in Fig. 3, the N-terminus of Factor B has two very striking regions of internal homology, each of approximately 60 amino acids, which are homologous to a third more distantly related sequence (Morley

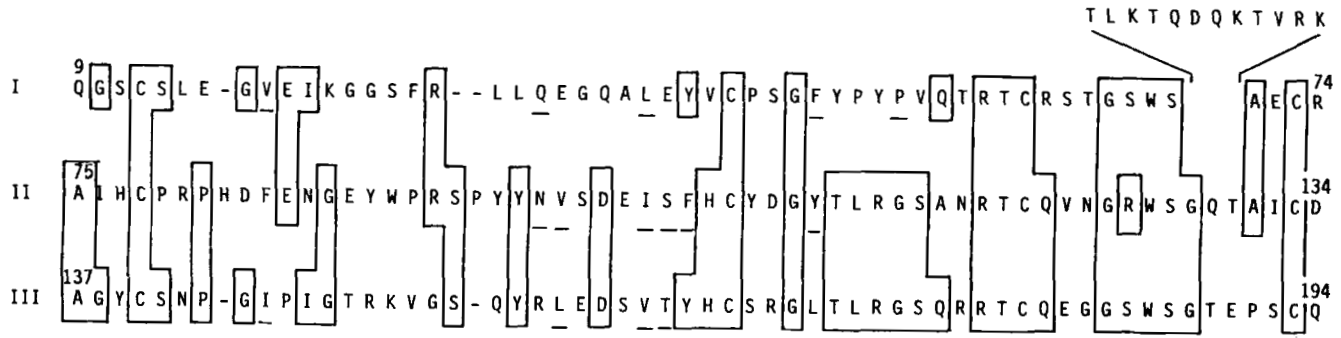

FIG. 3. Internal homologies of amino acid sequence in the Ba fragment of Factor B (Morley and Campbell, 1984). The three regions correspond to amino acids 9–74 (I), 75–134 (II), and 137–194 (III). Boxed areas represent identity and underlined regions represent functionally conserved amino acids designated by Dayhoff *et al.* (1972) from chemical similarity and accepted point mutation data and consist of (A,P,G), (N,Q), (D,E), (S,T), (C), (V,I,M,L), (K,R,H), and (F,Y,W).

and Campbell, 1984). Regions II and III, which have an overall homology of 47%, share 27 and 29% homology respectively with Region I. These internal repeats are unrelated to the repeating structures seen in the N-terminal regions of other serine proteinases such as prothrombin, plasminogen, and Factor IX (Jackson and Nemerson, 1980; Degen *et al.*, 1983; Anson *et al.*, 1984). Interestingly, however, these regions of Ba share pronounced sequence homology with similar repeating units in Factor H (Kristensen *et al.*, 1985), C4bp which has 8 repeats (Chung *et al.*, 1985a, b), and the unrelated plasma protein β_2-glycoprotein I which has 5 repeats (Lozier *et al.*, 1984). Determination of the gene structure of the Ba fragment has shown that the internal repeats are encoded in separate exons (see Fig. 5), suggesting that they arose by DNA duplication events. The finding of similar regions of homology in other proteins suggests that the regions may have evolved from a single primordial DNA segment. However, the relationship between the complement proteins and β_2-glycoprotein I, if any, is unclear.

The human Factor B cDNA has been used to screen a mouse liver cDNA library and to isolate Factor B cDNA clones from a mouse strain with the H-2k haplotype (Sackstein *et al.*, 1983). The largest insert isolated of 900 bp, pCmB5, was then used to screen a cDNA library prepared from another mouse strain of the H-2d haplotype, and a clone pBmB2 with an insert of 1.5 kb was identified. Sequence analysis of clone pBmB2 revealed that it encoded 94% of the murine Bb fragment and contained a 48 bp 3′ untranslated region and a poly(A) tail. The mouse Factor B mRNA, like its human counterpart, also displays the variant polyadenylation signal AUUAAA located 17 bases 5′ to the poly(A) tail. Comparison of the sequences of the Factor B cDNA clones from the H-2k and H-2d haplotypes revealed no nucleotide differences. In addition comparison of the human and mouse sequences revealed 83% nucleotide and amino acid sequence homology with no insertions or deletions, demonstrating the phylogenetic conservation of this MHC molecule.

Synthetic oligonucleotides and high complexity cDNA libraries have been successfully used to isolate cDNA clones containing inserts corresponding to C2. Bentley and Porter (1984) used a 17-base mixture of 64 synthetic oligonucleotides and isolated a clone pC201 with an insert of 400 bp. The insert was shown to code for a region near the C-terminus of C2 containing the active site Ser residue and the secondary substrate binding pocket of the serine proteinase domain. Woods *et al.* (1984) have also isolated C2 cDNA clones using a synthetic oligonucleotide, but in this case the complexity of the 17-base mixture was 576. Partial nucleotide sequencing of one of the clones with an insert of 500 bp established that it encoded a region at the N-terminus of the C2a fragment. To date the isolation of mouse C2 cDNA clones has not been reported.

B. Cloning of C2 and Factor B Genes

Cosmid clones containing the Factor B gene were isolated from a human genomic DNA library that contained the HLA Class III genes (Campbell and Porter, 1983; Carroll *et al.*, 1984a, b). Cosmids contain inserts of 35–40 kb and two overlapping clones covering 50 kb of DNA were characterized. Southern blotting and DNA sequence analysis established the position of the Factor B gene in the cosmids and showed that the gene is approximately 6 kb in length (Campbell and Porter, 1983; Campbell *et al.*, 1984; Morley and Campbell, unpublished). The C2 probe pC201 which is specific for a region near the 3′ end of the C2 gene was found to hybridize to restriction fragments derived from the same set of cosmids. The C2 probe site was mapped to a 2.6 kb region of DNA that lies ~500 bp from the 5′ end of the Factor B gene (Campbell *et al.*, 1984; Carroll *et al.*, 1984a). The 5′ end of the C2 gene was mapped by Southern blotting and DNA sequencing (Bentley *et al.*, 1985). A genomic fragment lying ~18 kb from the 5′ end of the Factor B gene was shown to hybridize to a mRNA species of ~2.9 kb which resembled the C2 mRNA, and a region of this fragment was sequenced and found to encode the N-terminus of C2. Thus the C2 gene spans 18 kb of DNA and this markedly contrasts the adjacent Factor B gene of 6 kb. The close homology of structure and function of C2 and Factor B suggests that they evolved from a common locus, probably by tandem duplication of the DNA. As the C2 and Factor B mRNA molecules of 2.9 kb (Bentley and Porter, 1984) and 2.6 kb (Morley and Campbell, 1984), respectively, are similar in length the marked divergence of the two loci will mainly be due to the length of noncoding sequences.

Further overlapping cosmids established the linkage of the Factor B gene with the C4 loci and showed that the 3′ end of the Factor B genes lies ~30 kb from the 5′ end of the C4A gene (Carroll *et al.*, 1984a, b) (Fig. 4). The alignment of the genes was confirmed by detailed Southern analysis of uncloned genomic DNA to verify linkage of adjacent probes.

Using the human cDNA probes for Factor B and C4 followed by chromosomal walking procedures, Chaplin *et al.* (1983) were able to isolate 18 overlapping clones spanning 240 kb of genomic DNA in the mouse S region (Fig. 4). A single Factor B gene was placed ~50 kb from two C4-like genes. DNA-mediated gene transfer later established that the C4-like gene lying closer to Factor B in fact encoded mouse Slp (Chaplin *et al.*, 1984). Although the C2 gene was not placed in this analysis in subsequent experiments it has been shown to occupy a similar position relative to the Factor B gene as its human counterpart (Perlmutter *et al.*, 1985).

The human Factor B gene is the first complement gene to have its structure completely determined (Campbell and Porter, 1983; Campbell *et al.*,

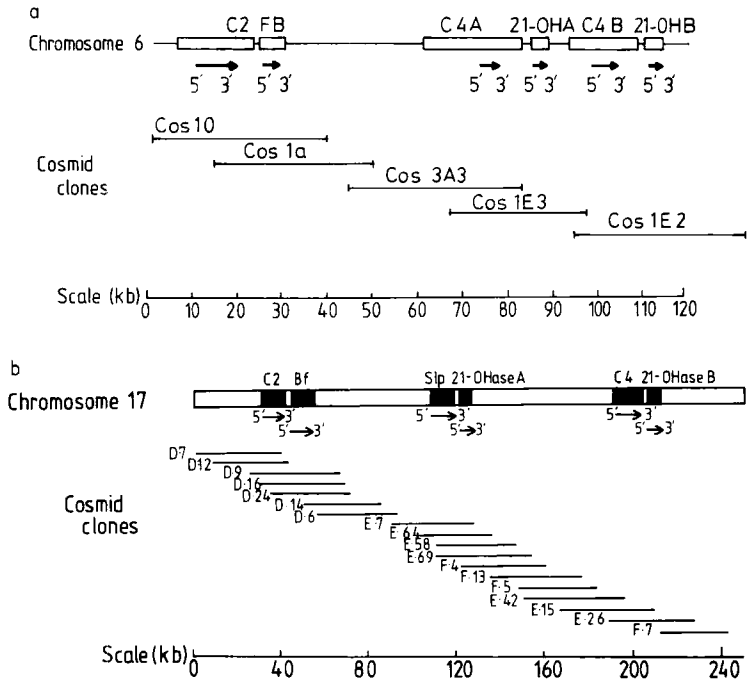

FIG. 4. Alignment of genes in the Class III region in HLA in man (Carroll *et al.*, 1984a,b, 1985b) (a) and in the S region in H2 in mice (b) (Chaplin *et al.*, 1983, 1984; White *et al.*, 1984a; Perlmutter *et al.*, 1985). Arrangement of cosmid clones used to align the genes is shown.

1984). It is 6 kb in length and is split into 18 exons as shown in Fig. 5. The Ba fragment of 30,000 M_r is encoded in 4 exons. Three of the exons (labeled I, II, and III in Fig. 5) encode the three repeat units making up the majority of this fragment and which have been discussed in a previous section. The catalytic peptide, Bb of 60,000 M_r, is encoded in 13 exons. The N-terminal half of Bb, which shows no homology with other serine proteinases except C2, is encoded in 5 exons and is that part of the molecule implicated in the binding to C3b in the C3 and C5 convertases (Smith *et al.*, 1984).

The C-terminal half of Bb is that part of the molecule homologous in structure and function to the catalytic chains of other serine proteinases (Christie and Gagnon, 1983). This section of the polypeptide chain is encoded by eight exons. The three active site residues His-501, Asp-551, and Ser-674 as well as the residues involved in the interaction with the substrate are contained in separate exons. The presence of these functionally important amino acids and binding regions in separate exons seems typical of the serine proteinase genes that have so far been described such as rat chymo-

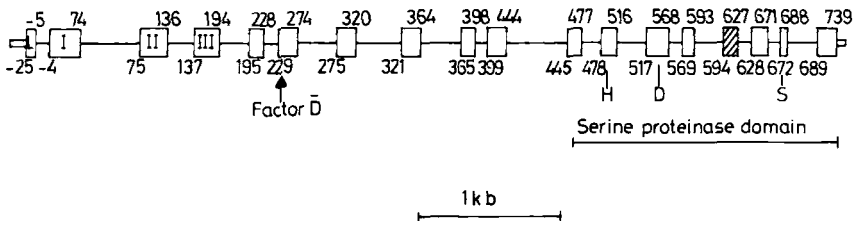

FIG. 5. Structure of the Factor B gene (Campbell *et al.*, 1984). Exons are shown boxed; the numbers refer to the amino acids encoded by each exon. L denotes the exon encoding 21 of the 25 amino acids of the putative leader peptide. The exons encoding the three homologous regions in Ba (see Fig. 3) are labeled I, II, and III. The position of the region encoding the Factor $\overline{D}$ cleavage site (between amino acids 234 and 235), which separates the Ba and Bb coding regions, is marked by the arrow. The eight exons encoding the serine proteinase domain are shown on the right of the figure. H, D, and S denote the positions of the codons for the active site residues His-501, Asp-551, and Ser-674, respectively.

trypsin (Bell *et al.*, 1984), rat elastase (Swift *et al.*, 1984), rat trypsin (Craik *et al.*, 1984), mouse kallikrein (Mason *et al.*, 1983), pig urokinase (Nagamine *et al.*, 1984), and human plasminogen activator (Ny *et al.*, 1984). Thus the active site and substrate specificity of these serine proteinases are produced by the joining of several exons each of which encodes a protein segment which is in itself catalytically inactive. One exception to this is the gene encoding human Factor IX where the C-terminal 181 amino acids, including the active site Asp and Ser residues, are encoded in a single exon (Anson *et al.*, 1984).

Comparison of the exon organization of this region of the Factor B gene with the exon organization of other serine proteinases such as trypsin, chymotrypsin, and elastase shows a correlation between them (Campbell and Bentley, 1985). However, the comparison has also revealed the presence of an exon in Factor B with no homologous counterpart in other serine proteinase genes (Campbell and Bentley, 1985). This exon encodes a region of the polypeptide chain (amino acids 594–627) lying between the active site Asp and Ser residues. Construction of a three-dimensional model of this domain of Factor B based on sequence homology with trypsin and chymotrypsin (Caporale *et al.*, 1983) has suggested that part of the polypeptide encoded by this exon may be over the active site of the molecule. This observation suggests that this section of the polypeptide may have some functional significance in the unique specificity and/or mechanism of activation of Factor B which differs from that of other serine proteinases. A similar region sharing 33% homology with residues 594–627 of Factor B has been found in C2 (Bentley and Porter, 1984; Gagnon, 1984; Campbell and Bentley, 1985) supporting the contention that this region may play some functional role in the activity of these two novel serine proteinases.

C. Restriction Fragment Length Polymorphism

C2 and Factor B are polymorphic and structural variants have been detected by differences in charge. The availability of cDNA probes for Southern blot analysis has provided an additional approach to that of protein typing for identifying polymorphism at the C2 and Factor B loci.

In the mouse Sackstein *et al.* (1984) have shown that the Factor B locus exhibits greater variability as detected by restriction fragment length polymorphism (RFLP) than that detected by protein typing. Various RFLPs have been defined which subdivide existing haplotypes and will be of value in defining further the genetic composition of the mouse MHC. These results are in contrast to the situation in man where in spite of a detailed analysis using in total 39 different enzymes (Woods *et al.*, 1984; Cross *et al.*, 1985), only one RFLP has been revealed using the Factor B cDNA as probe (Cross *et al.*, 1985). This DNA polymorphism, detected using the endonuclease *Taq*I, is characterized by the loss of a 4.5 kb fragment and the appearance of a 6.6 kb fragment. In 51 unrelated individuals examined, the 6.6 kb *Taq*I fragment was found associated with 39% of the F alleles, but not with the S, F_1, or S_1 alleles. The F allotype has been subdivided into two variants F^a and F^b by isoelectric focusing (Abbal *et al.*, 1985), and a correlation between the 6.6 kb *Taq*I fragment and the F^b subtype has since been established (Fathallah *et al.*, 1985). Further DNA polymorphism at the Factor B locus will no doubt be revealed when the nucleotide sequences of the various allelic variants have been compared.

In addition to the *Taq*I polymorphism which was mapped to the 3′ end of the C2 gene two further DNA polymorphisms have been defined at the C2 locus (Fig. 6). Bentley *et al.* (1985) observed a novel polymorphism using a 300 bp genomic fragment from a region near the 5′ end of the C2 gene. This probe in the majority of cases hybridized to an *Sst*I fragment of 2.7 kb. However, other fragments of 2.65, 2.6, and 2.4 kb were also detected. Mapping and family studies indicated that the different forms were inherited in Mendelian fashion and were derived from the same part of the genome, suggesting that they arose by several insertion/deletion events. Correlation of the *Sst*I fragments with Factor B types revealed that the 2.7 kb fragment was associated with all the S, F_1, and S_1 alleles, while among the F haplotypes approximately half were associated with the 2.7 kb fragment, with the remainder associated with the shorter size classes of *Sst*I fragment (2.4–2.65 kb) (Bentley *et al.*, 1985). The *Sst*I polymorphism was related to the *Taq*I polymorphism and to a third DNA polymorphism which was detected using the endonuclease *Bam*HI (Woods *et al.*, 1984; Bentley *et al.*, 1985), and is characterized by the loss of a 4.4 kb fragment and the appearance of a 6.6 kb fragment (Fig. 6). In a detailed analysis it was shown that the 6.6 kb *Bam*HI

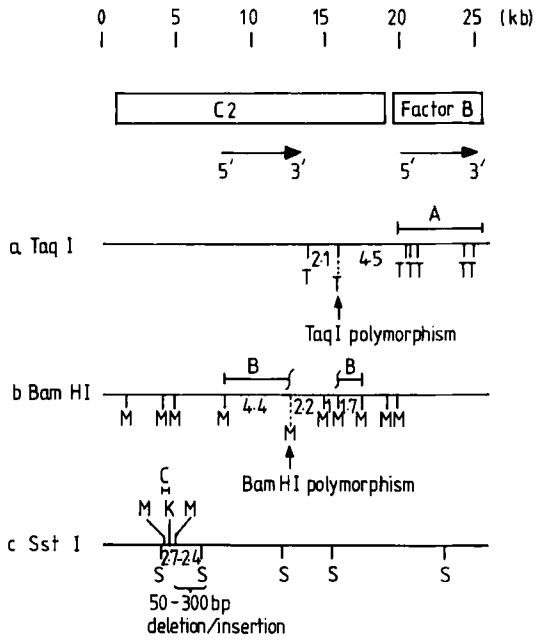

FIG. 6. The three DNA polymorphisms of the C2 gene (from Bentley *et al.*, 1985). The relationship of the three polymorphisms to one another is summarized in Table I. (a) The *Taq*I polymorphism, which is characterized by the loss of a 4.5 kb fragment and the appearance of a 6.6 kb fragment, was detected using the 2.3 kb Factor B cDNA as probe (Probe A) (Cross *et al.*, 1985). The probe hybridizes to the region of DNA shown by the solid bar. The arrow indicates the position of the polymorphic *Taq*I site. T, *Taq*I. (b) The *Bam*HI polymorphism, which is characterized by the loss of a 4.4 kb fragment and the appearance of a 6.6 kb fragment, was detected using a 594 bp C2-specific cDNA probe (Probe B) (Bentley *et al.*, 1985). The probe hybridizes to the region of DNA shown by the solid bar. The arrow indicates the position of the polymorphic *Bam*HI site. M,*Bam*HI. (c) The *Sst*I polymorphism was detected using a 300 bp genomic fragment (Probe C) prepared from a subclone of the 0.8 kb Bam HI fragment by a *Bam*HI/*Kpn*I double digest. The probe in the majority of cases hybridized to an *Sst*I fragment of 2.7 kb, but in a number of unrelated individuals fragments of 2.65, 2.6, and 2.4 were also detected (Bentley *et al.*, 1985). K, *Kpn*I; M, *Bam*HI; S, *Sst*I.

fragment was concomitant with the occurrence of the 6.6 kb *Taq*I fragment and the 2.4–2.65 *Sst*I fragments, while the 2.7 kb *Sst*I fragment cosegregated with the 4.4 kb *Bam*HI and 4.5 kb *Taq*I fragments (Table I) (Bentley *et al.*, 1985). As all individuals in this study were shown to carry the C2C allele these DNA polymorphisms markedly subdivide this allele when on the same haplotype as the F allele of Factor B (Bentley *et al.*, 1985).

The C2 cDNA probes have also been used to examine the molecular basis of C2 deficiency. In a study by Cole *et al.* (1985a) it was shown that the

TABLE I
ASSOCIATION OF THE C2 AND FACTOR B ALLELES IN UNRELATED INDIVIDUALS WITH THE POLYMORPHIC *Sst*I, *Bam*HI, AND *Taq*I FRAGMENTS

Allele		Size of fragment detected on haplotype (kb)			
C2	Factor B	*Sst*I	*Bam*HI	*Taq*I	Number of haplotypes
C	S	2.7	4.4	4.5	32
C	S_1	2.7	4.4	4.5	1
C	F_1	2.7	4.4	4.5	4
C	F	2.7	4.4	4.5	12
C	F	2.65	6.6	6.6	2
C	F	2.6	6.6	6.6	1
C	F	2.4	6.6	6.6	6

deficiency was not a result of a major gene deletion or rearrangement. Instead, when cultured monocytes taken from deficient individuals were examined no C2 mRNA was detectable suggesting that C2 deficiency results from a defect in transcription or posttranscriptional processing of C2 mRNA.

D. EXPRESSION OF C2 AND FACTOR B

Although the liver appears to be the principal site of synthesis of Factor B (Alper *et al.*, 1980), and probably also of C2, the extrahepatic synthesis of these two proteins and also of other complement proteins by cells of the monocyte/macrophage series has been observed (Colten *et al.*, 1979; Whaley, 1980; Beatty *et al.*, 1981). The importance of macrophage derived complement may be that it makes a significant contribution to the amount of complement present at tissue sites of injury or infection. The capacity to increase the concentration of complement components at these sites may be important in enhancing vascular permeability and chemotaxis which could lead to the further infiltration of phagocytic cells. Several studies have indicated that monocytes and macrophages display characteristic differences in C2 and Factor B synthesis as a function of maturation, tissue site, and response to stimuli that elicit an inflammatory response (Cole *et al.*, 1980, 1982, 1983; Colten, 1984). The availability of cDNA probes for C2 and Factor B has permitted preliminary analysis of the molecular mechanisms which control the expression of these two proteins in phagocytic cells.

Sackstein and Colten (1984) have studied the synthesis of Factor B in mouse peritoneal macrophages and have concluded that variation in Factor B production with time in culture is not a result of differences in catabolism, processing, or secretion of Factor B. Rather, it would appear that the variation is a function of differences in net amounts of Factor B mRNA. A similar

observation has also been made by Cole *et al.* (1985b) who have studied the synthesis of both C2 and Factor B in peripheral blood monocytes, and in tissue macrophages isolated from human breast milk and bronchoalveolar lavage. Again it was concluded that differences in the net production of C2 and Factor B were related to the level of C2 and Factor B mRNA in the different cell types and were not due to differences in rates of secretion, glycosylation, or catabolism. Taken together the results suggest that the regulation of production of C2 and Factor B occurs at a pretranslational level and may be due to increased transcription and/or differences in the rates of processing or catabolism of the two mRNA species. Distinct differences in the level of C2 mRNA in the various cell types was also evident. The level of C2 mRNA in bronchoalveolar macrophages was found to be 4-to-9-fold greater than in breast milk macrophages, whereas the level of Factor B mRNA varied 1-to-2-fold. This would suggest that despite their close linkage the expression of the C2 and Factor B genes is regulated independently in different cell types.

In order to examine further the expression of the C2 and Factor B genes, Perlmutter *et al.* (1985) have transfected mouse L cells with human and murine cosmid clones containing both genes. After DNA mediated transfer the expression of human and mouse Factor B and human C2 was evident as both C2 and Factor B mRNA, and the corresponding protein molecules, could be detected. The synthesis and secretion of Factor B appeared to be similar to that found in human and murine cells in primary culture. Similar experiments with C2 were made more difficult as the untransfected L cells synthesized and secreted murine C2. In cells transfected with the human cosmid in addition to a C2 mRNA species of ~2.6 kb a predominant C2 mRNA of ~2 kb was detected suggesting that the C2 gene was transcribed or the transcript modified in a slightly altered form compared to primary cells in culture. In addition multiple intracellular forms of C2 protein in transfected L cells similar, but not identical, to the multiple forms of C2 synthesized in Hep G2 cells (Perlmutter *et al.*, 1984) were also identified. In Hep G2 cells two of the forms remain cell associated while the third is secreted. Each of the forms appears to be derived from a separate primary translation product and could presumably be derived from distinct mRNA species. In addition to the secreted form of Factor B a membrane-associated form (Woo and Lachmann, 1981; Ooi and Ooi, 1982), which appears to differ in molecular weight from the plasma form (Ooi and Ooi, 1982), has also been reported. The presence of single C2 and Factor B genes would suggest that any differences in the primary structures of the secreted and cell-associated forms of the two proteins results from differential splicing of a precursor mRNA species or from posttranslational processing events. No function has been ascribed to the cell-associated forms of the two proteins.

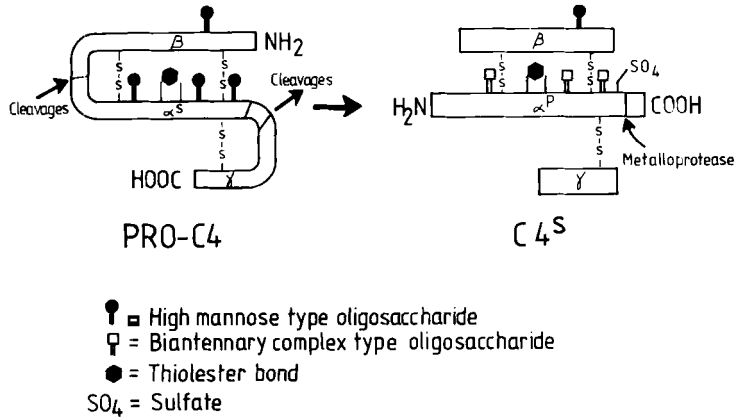

FIG. 7. Structural features of human C4 (Chan and Atkinson, 1985). Pro C4 is synthesized as a single peptide chain of 1722 amino acids and is processed by proteolytic removal of a leader peptide, short basic peptides between the β and α, α and γ chains to give the three chain structure and subsequently further hydrolysis of a peptide from the C-terminal end of the α chain. It is glycosylated by two types of polysaccharide on asparagine residues as shown, a thiol ester bond is formed and a sulfate residue is added.

III. C4

Activation of the complement system by the classical pathway is dependent on the presence in the serum of the fourth component (C4). Early studies defined this serum protein by its sensitivity to nucleophiles such as hydrazine or ammonia (Gordon *et al.*, 1926). This sensitivity since has been shown to be due to inactivation of an internal thiol ester which is important for covalent binding to the antigen–antibody complex (see Reid and Porter, 1981, for review).

C4 is activated by $C\bar{1}s$, which splits the α chain exposing a reactive acyl group which is available to form an ester or amide linkage with the immune complex (Fig. 7). This covalent linkage is important for stabilizing the C3 convertase leading to deposition of C3b which also binds covalently to the immune complex (for review see Müller-Eberhard and Schreiber, 1980). In addition to focusing assembly of the latter components, the covalent binding of C4 and subsequently C3 facilitates the processing and uptake of immune complexes (Schifferli *et al.*, 1980). A complete deficiency in one of the early components, i.e., C1q, C2, or C4 often leads to immune complex disease such as systemic lupus erythematosus (SLE) (Hauptmann *et al.*, 1985).

A. Structure of C4 Protein

The C4 protein is synthesized as a pro-molecule (pro-C4) of about 200,000 M_r (Hall and Colten, 1977; Roos *et al.*, 1978; Gigli, 1978) primarily by

hepatocytes (Saunders and Edidin, 1974) but also by macrophages in various tissues (Colten and Frank, 1972). However, the rate of synthesis and possibly regulation of C4 is different between the two cell types (Newell *et al.*, 1982; Rosa and Shreffler, 1983; Sackstein and Colten, 1984). Posttranslational modification of the single chain form results in glycosylation in the α and β chains (Gigli *et al.*, 1977; Bolotin *et al.*, 1977) and also a sulfate is added in the α4 region of the α chain (Fig. 7) (Karp, 1983). The pro-C4 molecule is processed into three disulfide-linked chains (Schreiber and Müller-Eberhard, 1974) which are ordered in the pro-molecule as β (75,000 M_r), α (95,000 M_r), and γ (30,000 M_r) (Goldberger *et al.*, 1980; Parker *et al.*, 1980). Processing by a plasmin-like enzyme (Goldberger and Colten, 1980) is probably coupled to secretion as the secreted form is found only in the extracellular medium of cell cultures of primary hepatocytes (Rosa and Schreffler, 1983). Further processing of the secreted form to the final plasma form results in removal of a 5000 M_r peptide from the C-terminal end of the α chain (Karp *et al.*, 1982a: Chan *et al.*, 1983) (Fig. 7).

B. Linkage of C4 to the MHC

Shreffler and Owen (1963) first mapped the gene for the control of murine C4 to the S region of the MHC on the basis of the protein level in the serum and they named the protein serum substance (Ss). In a search for allelic variants of Ss, a second form was identified by alloantisera and it was named sex-limited protein (Slp) since the variant was only observed in the serum of males in certain strains (Passmore and Shreffler, 1970).

The first indication for the function of the Ss protein came from studies by Demant *et al.* (1973) when they showed that the level of total complement activity was linked to the level of Ss protein in the serum and that addition of antisera specific for Ss reduced complement activity. This was followed by evidence of a serological cross-reactivity between the Ss protein and human C4 (Meo *et al.*, 1975; Curman *et al.*, 1975) and by a study suggesting that the Ss protein was bound to antibody-coated red cells that were treated in a manner to remove all components except C2 and C4 (Lachmann *et al.*, 1975). Demonstration that the Ss protein had C4 activity in a functional assay confirmed its identity (Carroll and Capra, 1978).

Linkage of the structural gene that encoded murine C4 was demonstrated by detection of a structural variant by peptide mapping and by linking the variation to the S region of the MHC (Carroll and Capra, 1979; Parker *et al.*, 1979). Demonstration that the Ss (C4) and Slp proteins were separate molecules was shown by immunoprecipitation and separation on SDS–PAGE (Roos *et al.*, 1978; Ferreira *et al.*, 1978). Analysis of the two proteins at the peptide level identified several differences that could not be explained by normal posttranslational processing and that suggested the proteins were products of two distinct loci (Parker *et al.*, 1979; Carroll and Capra, 1979).

Regulation of the S region loci is controlled both by testosterone and by

loci outside of the MHC. Expression of both C4 and Slp is regulated by testosterone levels in the mouse. The level of C4 in neonatal male mice increases with the level of testosterone to a maximum level at about 6 months, whereas female mice retain the level of juvenile males (Shreffler and Passmore, 1971). Similarly, the Slp protein levels increase with age in male mice of positive strains, however females, unless administered testosterone, do not normally express Slp (Passmore and Shreffler, 1971). The exceptions are three strains derived from wild mice, i.e., w 7, w 16, and w 19, in which females express a constitutive level of Slp (Hansen and Shreffler, 1976). Hormonal regulation of Slp levels appears to be by a cis-acting mechanism as (Slp-o × Slp-a)F_1s express only the Slp locus from the positive haplotype (Slp-a) (Parker *et al.*, 1981; Michaelson *et al.*, 1981). A possible molecular basis for this apparent escape in regulation will be discussed later. In addition to hormonal regulation, expression of Slp is regulated by two non-MHC loci, i.e., *regulation of sex limitation (rsl)*, which are trans-acting (Brown and Shreffler, 1980).

Genetic linkage of the serum level of C4 protein to the MHC in man was shown by mapping of a serum deficiency (Rittner *et al.*, 1975; Day *et al.*, 1975). With the demonstration that C4 variants could be detected by electrophoresis (Rosenfeld *et al.*, 1969), this technique was used to resolve multiple forms of the protein that subsequently were linked to the MHC on chromosome 6 (Teisberg *et al.*, 1976). Two common variants, F (fast) and S (slow), were proposed as the products of two separate but linked loci (O'Neill *et al.*, 1978a; Olaisen *et al.*, 1979). Improvement of the technique by pretreatment of serum samples with neuraminidase showed that both loci, i.e., C4-A (F) and C4-B (S), were polymorphic (Awdeh and Alper, 1980) with more than 35 alleles including null alleles at the two loci combined (Mauff *et al.*, 1983). MHC linkage of the two forms of human C4 was demonstrated independently when it was shown that the Chido and Rodgers red cell (RBC) antigens which were previously mapped to the MHC (Middleton *et al.*, 1974; Giles *et al.*, 1976) were fragments of the α chain, i.e., C4d (O'Neill *et al.*, 1978b; Tilley *et al.*, 1978). The C4d fragment, which is about 44,000 M_r, is released from the α chain on inactivation of C4b by Factor I and C4bp (Reid and Porter, 1981). Similarly, in the mouse, the red cell antigen H-2.7 was identified as a C4d fragment of the C4 protein (Ferreira *et al.*, 1980).

C. Functional Activity of C4 Allotypes

Both forms of the C4 protein in man, i.e., C4-A and C4-B, are functionally active, however the C4-B protein is several fold more active in the hemolytic assay (Teisberg *et al.*, 1977). This difference in activity has been explained as due to a difference in efficiency of covalent binding to the antibody-coated RBC (Law *et al.*, 1984; Isenman and Young, 1984). The C4-A protein was found to bind more efficiently to protein antigens, and the C4-B protein was found to bind more efficiently to carbohydrate antigens. The difference

between the two classes of C4 was greater when reaction rates with small molecules was measured. A C4-A allotype reacted 300 times more rapidly with the amino group of glycine than a C4-B allotype and the C4-B 10 times more rapidly with the hydroxyl group of glycerol than C4-A (Dodds *et al.*, 1985).

An even more striking difference in the hemolytic activity of the C4 allotypes is found in C4-A6 which has been reported to have little or no hemolytic activity (O'Neill *et al.*, 1980; Teisberg *et al.*, 1980). When C4-A6 was isolated by affinity chromatography and its covalent interaction with antibody-coated red cells was measured it was found to be similar to that of other C4-A allotypes and several fold less than that of the C4-B allotypes (Dodds *et al.*, 1985). Similarly all the C4-A allotypes reacted equally with amino and hydroxyl groups. Activation by $C\bar{1}s$ and inactivation by Factor I and C4bp were the same for all the allotypes and the C3 convertase activity was the same if the number of C4 molecules bound to the antibody red cells

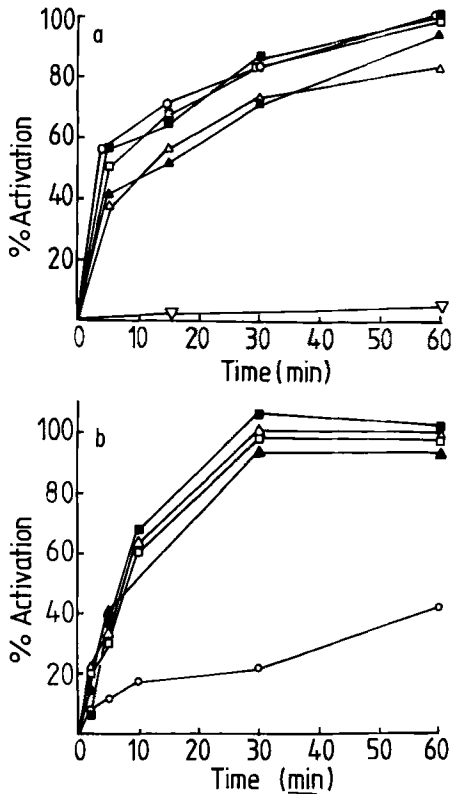

FIG. 8. A comparison of the C3 convertase, $C\overline{42}$ (a), and the C5 convertase, $C\overline{423}$ (b) activities of convertases formed from the same C2 and C3 proteins, but different C4 allotypes, on antibody coated red cells. C4 allotypes: (■) A3, (△) A4, (○) A6, (□) B1, (▲) B2, (▽) control with no C4. In each case about 3000 molecules of C4 were bound to the red cell.

was kept constant. The very low hemolytic activity of C4-A6 was found to be due to low C5-convertase activity when this allotype was used to form this complex protease $C4\overline{23}$ (Fig. 8). As the C3 convertase activity was the same, the difference probably lies in the covalent binding of C3 to the complex which with the C4-A6 allotype forms a less efficient protease. When the derived amino acid sequence of C4-A6 is obtained, this should identify the section of the C4 molecule involved in this interaction.

The exceptional polymorphism of C4 and the big differences in hemolytic activity of the isotypes led to the suggestion that this may give a biological advantage in the lysis and removal of a range of pathogens differing widely in surface structures. It could also contribute to the association of particular haplotypes in this section of HLA with susceptibility to immune complex disease and to autoimmune disease (Porter, 1983).

In the mouse, only one form of the C4 is active in the hemolytic assay (Ferreira *et al.*, 1978). The Slp protein is not active in the hemolytic assay, is not cleaved by $C\overline{1}s$ (Ferreira *et al.*, 1978) and the secreted form is not processed to the final plasma form (Karp *et al.*, 1982a). However, the protein does have an internal thiol ester which is intact based on reactivity with methylamine (Karp *et al.*, 1982b).

D. Molecular Cloning of C4 cDNA

The cloning of DNA complementary to the C4 mRNA isolated from either human (Carroll and Porter, 1983; Whitehead *et al.*, 1983) or mouse (Ogata *et al.*, 1983) liver has proved very useful in extending the genetics to the molecular level and in determining the structure of this important protein. Nucleotide sequence analysis of a cDNA clone, pAT-A, which contained the complete coding sequence showed that the N-terminus of the β chain was preceded by a leader sequence which was later confirmed in a genomic clone to be 19 amino acid residues in length (Belt *et al.*, 1984, 1985). The murine C4 is also transcribed as a pre-pro molecule with a 19 residue leader sequence (Sepich *et al.*, 1985). At the junctions between the β and α and the α and γ chains are stretches of basic residues. In man, the basic sequence Arg-Lys-Lys-Arg immediately preceding the N-terminus of the α chain probably is removed during intracellular processing as the C-terminus of the β chain is Lys-Thr-Thr (Gagnon and Law, unpublished). A similar basic region, i.e., Arg-Gln-Lys-Arg, was found at the β–α junction in the mouse (Nonaka *et al.*, 1984). While the C-terminus of the α chain has not been reported, it is probable that the tetraarginine residues immediately preceding the N-terminus of the γ chain are removed in both man (Whitehead *et al.*, 1983; Belt *et al.*, 1984) and in mouse (Ogata *et al.*, 1983) as has been proposed for murine C3 (Domdey *et al.*, 1982).

E. Molecular Basis of the Two C4 Isotypes

The molecular basis for the difference between the two isotypes of human C4 was provided by comparison of the two cDNA clones pAT-A and pAT-F

which represent the complete C4-A and near complete C4-B coding sequences, respectively (Belt *et al.*, 1984, 1985). The cDNA clones were shown to represent C4-A and C4-B based on homology with the partial amino acid sequence of the two forms by Hellman *et al.* (1984). Lundwall *et al.* (1981) demonstrated that large fragments representing about two-thirds of the C4d region could be separated from C4 isolated from pooled serum and that these fragments, i.e., Tryp-C4d, could be identified as either C4-A or C4-B using antisera specific for the two isotypes. Analysis of the Tryp-C4d fragments, which were about 28,000 (C4-A) and 30,000 (C4-B) in apparent M_r, by amino acid sequencing showed that they were identical except at five positions (Hellman *et al.*, 1984). The difference in apparent molecular weight of 2000 was proposed to be the result of a conformational change since carbohydrate was not present and the two fragments were identical in total number of residues (Hellman *et al.*, 1984). This difference in size probably accounts for a similar mobility difference between the α chains of C4-A and C4-B seen on SDS–polyacrylamide gels (Roos *et al.*, 1982).

Comparison of the nucleotide sequences of C4-A and C4-B showed a marked conservation. Out of more than 4600 nucleotides compared only 14 differences were observed. Of the 14 substitutions, 11 resulted in codon changes and 9 of the changes were clustered in the C4d region (Belt *et al.*, 1984). Each of the other two codon changes were located either in the β or the γ subunits. Six of the nine amino acid substitutions, which were derived from the nucleotide sequence, were proposed as isotypic differences based on the sequence results of Hellman *et al.* (1984) described above and on the analysis of an additional four clones that represented C4-A or C4-B (Table II) (Belt *et al.*, 1984, 1985). These six amino acid substitutions may provide a molecular basis for the functional, serological, and electrophoretic differences between the two forms.

Law *et al.* (1984) have proposed that the substitution of the basic residues, i.e., His for Asp (position 1106) and Arg for Leu (position 1191), in C4-B may account for the increase in binding efficiency to hydroxyl groups relative to C4-A, as these substitutions could increase the nucleophilicity of alcohols. The Asp instead of Arg at position 1106 could facilitate deprotonization of amino groups and increase their reactivity with the C4-A protein. The Pro instead of Leu at position 1101 probably would alter the conformation of C4-A in the region of the thiol ester and thus affect the relative binding efficiency. It is interesting that in the homologous region, the mouse C4 sequence encodes the C4-A-like residues at position 1101 and 1102, i.e., Pro and Cys, and C4-B-like residues at positions 1105, 1106, and 1191, i.e., Ile, His, and Arg (Sepich *et al.*, 1985). Since there is only one active form of C4 in the mouse, it would not be surprising if the mouse C4 protein bound to both carbohydrate and protein antigens with an equal efficiency.

The two isotypes of murine C4, i.e., C4 and Slp, are not as conserved as

C4-A and C4-B. Comparison of cDNA clones that encode the complete coding sequences showed an overall homology in the three chains as 96% (β), 94% (α), and 97% (γ) (Sepich *et al.*, 1985; R. T. Ogata, unpublished data). However, the differences appeared to be clustered and might explain some of the known differences between C4 and Slp. The two isotypes were distinguished by hybridizing short probes of about 100 bp from the C-terminal region of the α chains to filters containing RNA from strains of mice that synthesized both C4 and Slp and strains that synthesized only C4 (Ogata and Sepich, 1984). Alternatively, C4 and Slp sequences were distinguished by analyzing cDNA clones isolated from a library prepared from a strain that synthesized only C4 (Nonaka *et al.*, 1984).

Unlike C4, Slp does not appear to be activated by $C\bar{1}s$ as discussed above. Analysis of the region where a peptide bond is hydrolyzed releasing the N-terminal C4a peptide showed that Slp like C4 encodes Arg. However, immediately following Arg, 10 of the next 13 residues were different. Nonaka *et al.* (1984) have proposed that these substitutions, which included three acidic residues, might alter the protein conformation such that the α chain would not be cleaved by $C\bar{1}s$.

A second cluster of differences in the C-terminal region of the α chain where 5 out of 15 residues were substituted might account for the apparent failure of the secreted form of the Slp α chain to be converted to the plasma form (Ogata and Sepich, 1984; Tosi *et al.* 1984).

Analysis of the N-terminus of C4d showed that the Slp sequence encoded Gly-*Gln*-Met while in C4 of man and mouse the sequence is Gly-*Arg*-Thr where the Arg peptide bond is cleaved during inactivation by Factor I and C4bp. This suggested that the Slp α chain would not be cleaved normally (Nonaka *et al.*, 1984).

In the thiol ester region, both C4 and Slp have the sequence Cys-*Ala*-Glu-Gln which differs from that of human C4 which has a *Gly* following the Cys (Campbell *et al.*, 1981; Harrison *et al.*, 1981). The two residues preceding the Cys were different, C4 encoded Gln-Gly while Slp encoded Arg-Ser. The significance of these substitutions is not clear as the Slp thiol ester is intact.

F. Molecular Basis of C4 Allotypes

Human C4 is highly polymorphic based on protein electrophoresis with more than 35 alleles at the two loci (Mauff *et al.*, 1983) of which 15 occur in the population at a frequency of 1% or greater (Schendel *et al.*, 1984). While the structural basis for the polymorphism was not known, the electrophoretic variation was localized to the C4d region by analysis of the C4c and C4d fragments in two-dimensional gel electrophoresis (Mevag *et al.*, 1981). Analysis of the individual chains of C4 from various strains of mice by peptide

TABLE II
PROPOSED C4 ISOTYPIC AND ALLOTYPIC DIFFERENCES

Position	C4 Allotype				
	A3[a]	A4[a]	B1a	B1b	B2
1054	Asp	Asp	Gly	Gly	Asp
1101[b]	Pro	Pro	Leu	Leu	Leu
1102[b]	Cys	Cys	Ser	Ser	Ser
1105[b]	Leu	Leu	Ile	Ile	Ile
1106[b]	Asp	Asp	His	His	His
1157	Asn	Asn	Asn	Ser	Ser
1182	Thr	Ser	Thr	Thr	Thr
1188[b]	Val	Val	Ala	Ala	Ala
1191[b]	Leu	Leu	Arg	Arg	Arg
1267	Ser	Ser	N.D.[c]	N.D.	Ala

[a] Tentative assignment.
[b] Indicates proposed isotypic positions.
[c] N.D., not determined.

mapping has identified only limited polymorphism (Carroll and Capra, 1979; Parker *et al.*, 1979). However, separation of intact C4, using an electrophoretic technique similar to that used in the study of human C4, has identified seven allotypes among both strains of inbred mice and wild mice (Natsuume-Sakai *et al.*, 1980). It was not determined if the structural variation was clustered in a single region as found in allotypes of human C4.

The structural basis for polymorphism of human C4 has been proposed for at least four different allotypes, i.e., C4-A4, C4-A3, C4-B1, C4-B2, as well as a split of the C4-B1 allotype into two subtypes (Table II) (Belt *et al.*, 1985). Unlike the complex polymorphism observed for Class I (Steinmetz and Hood, 1983) and Class II (Kaufman *et al.*, 1984), the C4 allotypes may be explained by a single base change resulting in a coding substitution. The proposed allotypic substitutions were based on comparison of three cDNA clones that were isolated from a library prepared from liver mRNA of an individual heterozygous at both C4 loci, i.e., C4-A3,4/C4-B1,2. Two of the clones represented C4-A alleles and differed by only one nucleotide, i.e., Thr (C4-A3) instead of Ser (C4-A4), for the region compared.

The three C4-B allotype differences described in Table II were based on comparison of the C4-B cDNA clone with three homologous 0.9 kb genomic fragments isolated from two separate libraries of genomic DNA that represented either homozygous C4-B1 or heterozygous C4-B1,B2 (Belt *et al.*, 1985). It is interesting that the mouse C4 sequence was identical to the C4-B2 allotype at each of the four allotypic positions (Table II).

Sepich *et al.* (1985) have compared the nucleotide sequence of C4 in two regions, i.e., a 465 bp region that included the β–α junction and the C4a peptide and a 360 bp region that included the thiol ester site, of two strains of mice, i.e., w 7 and FM. The thiol ester region compared did not extend to the region homologous to the human C4d that includes the polymorphic positions. They identified substitution at 10 positions, i.e., about 1% difference, of which 5 resulted in codon changes. Given the greater degree of divergence between C4 and Slp (about 5%) than the two C4 allotypes, it was surprising that 9 of the 10 positions where C4 w 7 differed from C4 FM, that the Slp Fm sequence was identical to C4 w 7. A similar observation was made on comparison of the allotypic positions for human C4-A and C4-B. For example, at position 1182, C4-A4 is characterized by a Ser, however C4-A3 and each of the three C4-B alleles have a Thr at this position (Table II). This pattern of variation could be explained by a mechanism such as gene conversion in which DNA is exchanged between two nonallelic loci as proposed for the polymorphism observed in Class I alleles (Weiss *et al.*, 1983). Alternatively, the identity between the two nonallelic loci at positions where allotypic differences occur may represent divergence from an ancestral gene. This latter argument would explain the similarity between the human C4-B2 allele and the murine C4 as mentioned above.

G. Molecular Map of the MHC Class III Region

This region (Fig. 1) represents about 0.7 cM (Olaisen *et al.*, 1983) or about 1000 kb. In the mouse, the gene controlling the level of functional activity of C2 (Gorman *et al.*, 1980) and structural genes encoding C4 and Slp have been mapped to the homologous region of H-2. This region was first defined as the S region and maps between the H-2 I and D regions. The distance has been estimated as about 0.11 cM on the basis of genetic recombination (Snell *et al.*, 1976), however these estimates should be interpreted with caution as there may be specific regions where recombination occurs at a relatively high frequency. Such a region or "hot spot" has been identified in the mouse Class II region (Steinmetz and Hood, 1983).

An equivalent Class III region probably exists in all mammalian species as well as in birds. In the guinea pig, deficiency of the C4 protein has been mapped to the MHC (Shevach *et al.*, 1976) and inherited polymorphism of guinea pig Factor B has been linked to C4 (Bitter-Suermann *et al.*, 1977). Genetic polymorphism and a total deficiency in C2 in the guinea pig have been mapped to the MHC (Bitter-Suermann *et al.*, 1981). In the chicken, total serum complement activity has been linked to the MHC (Chanh *et al.*, 1976).

The arrangement of genes encoding the complement proteins in the MHC has been determined by ordering of overlapping cloned genomic fragments

(Carroll *et al.*, 1984a, b; Chaplin *et al.*, 1983) (Fig. 4). In man, the C2 and Factor B genes were separated by about 500 bp and were about 30 kb away from the two C4 genes which were separated by about 10 kb. The two C4 loci probably arose from a single duplication event of about 30 kb that included the C4 gene and 6 kb of 3′ flanking region. All four complement genes were oriented in the same direction. The C4-A and C4-B genes were identified and ordered in the cosmid clones by hybridization with synthetic oligonucleotides prepared to include the region where the isotypic differences were clustered (Carroll *et al.*, 1984b). The identity of the genes was confirmed later by nucleotide sequencing of this region (Belt *et al.*, 1985). Analysis of the region flanking the 5′ ends of the C4-A and C4-B genes using short DNA probes showed that these regions were not duplicated. Expression of cos 3A3, which includes a C4-A gene (Fig. 4), and cos KEM 1, which includes a C4-B gene, after transfection of LTK^- cells with the cosmid clones demonstrated that these cloned fragments encoded hemolytically active proteins (K. T. Belt, unpublished).

Duplication of the C4 loci in the mouse included a larger segment of DNA, i.e., about 55 kb (Chaplin *et al.*, 1983), and the two loci were separated by about 80 kb compared to 10 kb in man (Fig. 4). The Slp gene was identified as closest to the Factor B gene by hybridization of genomic DNA with short cDNA probes isolated from the C-terminal region of the C4 and Slp α chains (Ogata and Sepich, 1984). Unlike in man, the flanking regions 5′ to Slp and C4 were duplicated, however, a region of unique DNA separated the two genes (Chaplin *et al.*, 1983).

A gene for steroid 21-hydroxylase (21-OHase) has been identified less than 3 kb from the 3′ end of each C4 locus in both man (Carroll *et al.*, 1985a; White *et al.*, 1985) and in mouse (White *et al.*, 1984a) (Fig. 4). This region probably was duplicated by the same event as the two C4 genes. While the two genes appeared to be similar by restriction mapping only one seems to be active in the adrenal gland (White *et al.*, 1984b). In man, the 21-OHase B gene is the important gene for steroid biogenesis (White *et al.*, 1985); whereas, in the mouse the 21-OHase A gene is the active gene in the adrenal tissue (D. Chaplin, and K. L. Parker, unpublished data).

An important question is whether the duplication of the two C4 genes occurred prior to separation of the species. The argument in support of duplication occurring after separation is supported by the results of structural studies and by the observation that not all mammals appear to have duplicated loci. The overall homology between the complete coding sequence of human C4-A and the murine C4 is of the order of 80% at the nucleotide level (Sepich *et al.*, 1985; R. T. Ogata, unpublished). However, the homology between C4-A and C4-B is greater than 99% (Belt *et al.*, 1984, 1985) and the homology between murine C4 and Slp is about 95% (R. T.

Ogata, unpublished). The simplest interpretation of these results is that the loci duplicated after separation of the species. Studies using C4 DNA probes suggest that both the guinea pig (Whitehead *et al.*, 1983) and the hamster (Levi-Strauss *et al.*, 1985) have only a single C4 locus. These results would support the argument that duplication occurred independently in those species with two C4 loci.

H. Polymorphism in the Number of C4 Genes Expressed

Genetic studies in man have demonstrated that deficiency in the serum of either C4-A or C4-B is relatively common. This deficiency was linked to the MHC and defined as a null allele (QO) with a gene frequency of 10–15% (Hauptmann *et al.*, 1985; Schendel *et al.*, 1984). The molecular basis for the deficiency was not known, but Raum *et al.* (1984) proposed that the null alleles resulted from unequal crossover. Thus, some chromosomes would have only a single C4 locus and others would have three loci encoding C4. Duplication has been demonstrated at the protein level and the gene frequency has been estimated as 1–2% (Bruun-Peterson *et al.*, 1982; Raum *et al.*, 1984; Uring-Lambert *et al.*, 1985). In the mouse, the Slp locus, which is regulated by testosterone in most strains, is not expressed in male mice of certain strains. Thus, only a single locus for C4 is expressed on some chromosomes in mouse.

Understanding the molecular basis for the null alleles may be clinically important, as the C4-AQO allele has been shown to occur in individuals with SLE at 2- to 3-fold greater frequency than in normal individuals (Fielder *et al.*, 1983). To determine the basis for the null alleles at the DNA level, Southern analysis was used. Genomic DNA representing at least 18 different haplotypes with a null allele at the C4-A locus and 6 with a null allele at the C4-B locus (Carroll *et al.*, 1985b) was examined. The results from this study suggested that most haplotypes had two C4 genes. However, two haplotypes were identified that had a deletion of the C4-A gene (Fig. 9) and one haplotype that had a deletion of the C4-B gene (Fig. 10).

Unequal exchange of genomic DNA between sister chromatids during meiosis would explain both the deletions and the finding of at least one haplotype with a duplicated C4-B locus (Carroll *et al.*, 1984b). The degree of homology between the coding sequences of the nonallelic loci would favour pairing of C4-A and C4-B genes from separate chromatids. Analysis of intron sequences in the C4d region has shown that the C4-A and C4-B noncoding sequences, at least in this region, are also highly conserved with less than 1% difference observed (Belt *et al.*, 1985). The 6–7 kb intron near the 5′ end of the C4-A gene although absent in one C4-B gene (Carroll *et al.*, 1984b) is present in others (A. Palsdottir, unpublished).

In order to look for evidence of recombination that would explain deletion

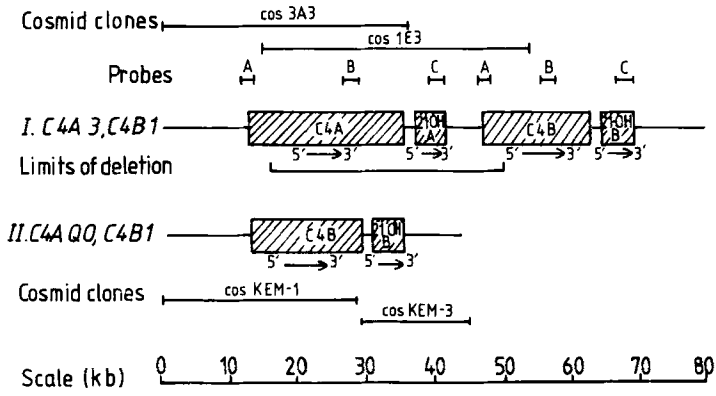

FIG. 9 Comparison of C4 haplotypes C4-A3, C4-B1 and C4-AQO, C4-B1 in man. The C4-A gene is deleted on some chromosomes (Carroll *et al.*, 1985b).

of the C4-A gene, a cloned genomic fragment that included the single C4-B gene was isolated from a library of the C4-A null haplotype (Carroll *et al.*, 1985b). The single C4 gene was identified as C4-B by nucleotide sequencing of the region containing the isotypic differences (Belt *et al.*, 1985). Comparison of the region flanking the 5′ end of the gene by restriction mapping of subcloned genomic fragments with the homologous region of the normal C4-A gene confirmed that the C4-A gene was deleted (Carroll *et al.*, 1985b). Because of the homology in this region the precise site of recombination could not be established by restriction mapping. However, analysis at the nucleotide level might identify the site of recombination. Since the molecular maps of the two haplotypes with a deletion of the C4-A gene were identical, it is likely that the mechanism for deletion was similar.

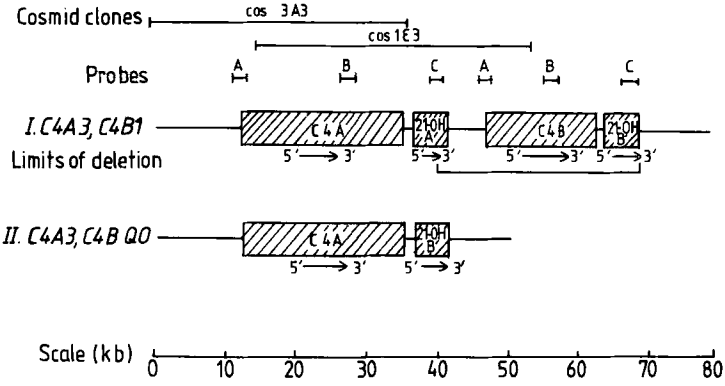

FIG. 10. Comparison of C4 haplotypes C4-A3, C4-B1 and C4-A3, C4-BQO in man. The C4-B gene is deleted on at least one chromosome (Carroll *et al.*, 1985b).

Occurrence of null alleles in the population is relatively common as discussed above, yet only a few of the haplotypes with null alleles were due to deletions. While failure to express a gene product could be the result of one of a variety of defects, an alternative might be that the null allele has been converted to the same isotype as the locus that is expressed. Thus, rather than a single C4-A locus and a C4-B null, perhaps both nonallelic loci encode for a similar C4-A protein. This would be difficult to detect at the protein level unless the two C4-A products could be distinguished in some manner such as by electrophoresis. Haplotypes with the phenotype of duplicated C4-A locus, e.g., C4-A3, C4-A2, C4-BQ0, and null at the C4-B locus have been identified (Raum *et al.*, 1984; Uring-Lambert *et al.*, 1985). These haplotypes were interpreted as having a duplication of the C4-A locus, although they may actually represent a conversion of the C4-B locus to C4-A (A. Palsdottir, unpublished). This hypothesis can be tested by characterizing these haplotypes at the DNA level as described for the analysis of the C4-A null haplotype (Carroll *et al.*, 1985b).

Null alleles at the Slp locus in at least two strains of mice have been analyzed by the Southern technique and were shown not to be due to missing genes (Levi-Strauss *et al.*, 1985). However, duplication of C4-like genes in at least three strains of mice has been observed (Tosi *et al.*, 1984; Levi-Strauss *et al.*, 1985). Levi-Strauss *et al.* (1985) have reported that the wild-derived strains in which the females express a constitutive level of Slp, i.e., w 7, w 16, and w 19, have two additional bands that hybridize with the C4 DNA probes. The w 7 strain that produces higher levels of Slp protein than the other two, appears to have a third extra gene based on differences in intensity of hybridization. These additional C4-like genes were identified as Slp by hybridization with short probes that distinguish C4 and Slp (Rosa *et al.*, 1985). They proposed that the w 16 and w 19 strains had two additional Slp genes and that the w 7 strain had a third Slp gene. As an explanation for the expression of Slp protein by females in these strains, Rosa *et al.* (1985) proposed that the duplicated Slp gene may have acquired the C4 controlling element through a conversion type event. Unequal crossover resulting in a hybrid C4/Slp gene would explain both the multiple copies of Slp and the constitutive expression by females. Further analysis of these strains at the genomic level may provide an understanding of the regulation of Slp expression by testosterone levels.

IV. C1q

The first component of complement, C1, is a complex of 1 molecule of C1q and 2 molecules each of C1r and C1s. Electron microscopy showed C1q to have a most unusual shape, the bunch of tulips structure, with 6 fibrous

sections held together in the lower half and with globular heads on the upper end of the fibres (Knobel *et al.*, 1975). It has a molecular weight of 460,000 and contains 18 peptide chains 6A, 6B, and 6C each with an N terminal collagen region of 81 amino acid residues and a C terminal globular section of 145 residues (Reid, 1983). C1q interacts with the Fc domains of the aggregated antibody through the globular head and with the $C1r_2$–$C1s_2$ dimer through the collagenous section. These interactions lead to activation of C1r and then of C1s by $C\bar{1}r$.

The genetics of C1q is of interest first because there is evidence of two structurally different forms of C1q (Reid and Solomon, 1977). Second, information might be gained of the evolution of this unusual half collagen-half globular structure and of its relation to collagen, and third, clarification might be possible of inherited defects of C1q which cause immune complex type of disease.

A synthetic oligonucleotide probe based on the known amino acid sequence of human C1q B chain (Reid, 1983) was used to obtain cDNA clones from a library prepared from liver mRNA. Sequencing of the cDNA inserts confirmed the amino acid sequence with the correction of one position and were used to isolate a clone from a human genomic cosmid library. Sequencing of this clone showed that the B chain gene had a single intron as shown in Fig. 11 (Reid, 1985). This organization is different from that of fibrillar collagen genes which generally have exons of 45, 54, 99, 108, or 162 base

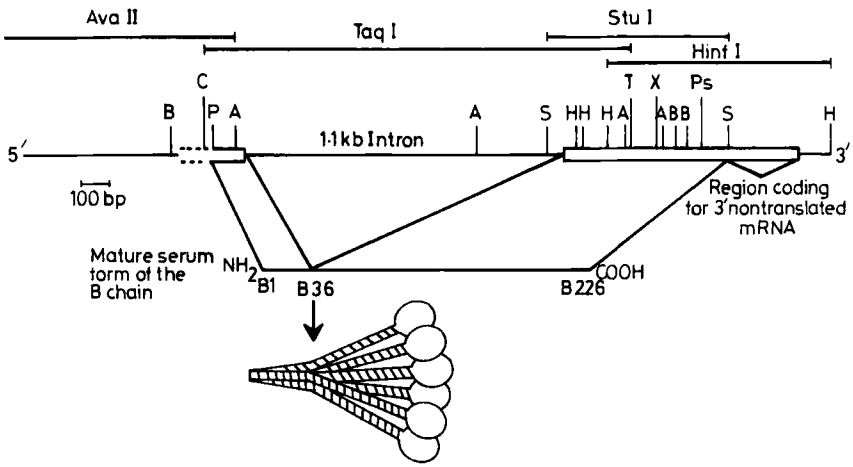

FIG. 11. Restriction maps of the C1q B chain gene (Reid, 1985). Restriction enzyme site A, *Ava*II; B, *Bst*E1I; C, *Cla*I; H, *Hin*fI; P, *Pvu*II; Ps, *Pst*I; S, *Stu*I; T, *Taq*I; X, *Xho*I. The solid boxed regions show definitely established exon sequence. The 5′ exon has not yet been defined. B36 is the position of the glycine residue corresponding to the break in the X-Y-Gly repeat sequence of the collagen section of the B chain.

pairs corresponding exactly to repeats of the X-Y-Gly amino acid triplet sequence (Chu *et al.*, 1984; Yamada *et al.*, 1984). In the B chain gene, the intron comes within the codon for glycine at position 36, about the middle of the collagen sequence where there is a discontinuity in the X-Y-Gly repeated triplet in all three chains. This break is probably responsible for the bend which occurs in the collagenous fiber section. Only one B chain gene has been detected so far and as the 3′ sequence is complete and corresponds with the expected C terminal amino acid sequence any different forms of C1q which occur are likely to arise from differences in splicing at the 5′ end of the gene. The sequence here is, however, incomplete both for the cDNA and the genomic DNA. Clones for the A and C chains have not yet been obtained.

Somatic cell genetics using the cDNA clone placed the C1q B chain on chromosome 1 (Solomon *et al.*, 1985).

V. C3

C3 is the most abundant of the complement components occurring at about 1.5 g/liter. It occupies a central position in the complement cascade (Fig. 2), it plays an important role in the opsonization of antibody–antigen aggregates, and peptides released in the activation and inactivation of C3 have anaphylatoxic and other activities. Individuals unable to synthesize C3 suffer repeated bacterial infections (reviewed in Lachmann, 1984).

Mouse C3 was the first complement component for which cDNA clones were obtained (Domdey *et al.*, 1982) and Fey and colleagues have now established the complete cDNA sequence of mouse (Lundwall *et al.*, 1984; Fey *et al.*, 1984; Wetsel *et al.*, 1984) and also of human C3 (Bruijn and Fey, 1985). There is 79% identity of nucleotide sequence and 77% of amino acid sequence between the two species. C3 is synthesized as a single precursor polypeptide of 1663 amino acid residues in the human protein and is cut into two chains before secretion. 22 Residues of pro C3 form the signal peptide, 645 the β chain, a tetraarginine sequence is lost in processing and 992 residues form the α chain. Very similar figures were found for the mouse C3. The 27 half-cystine residues are identical in both species and the sequence around the reactive thiol ester bond near the center of the α chain is highly conserved. The thiol ester bond is exposed when C3 is activated by C3 convertase and forms a covalent ester or amide bond with the activating substances (reviewed in Reid and Porter, 1981).

The structure of the C3 gene is not yet available, but its size is estimated to be 24 kb (Wiebauer *et al.*, 1982). There will be particular interest in the 5′ region, presumably including the promotor region, as C3 is an acute phase protein, the concentration of which rises rapidly in inflammatory conditions.

VI. C5

C5, as C3 and C4, is synthesized as a single chain precursor molecule subsequently split into a two polypeptide chain protein similar to C3. The three proteins have homologous structures and, in each, activation releases a biologically active peptide of about 80 amino acid residues from the α chain, but C5 lacks the intrachain thiol ester bond (Law *et al.*, 1980; DiScipio, 1981). A partial cDNA sequence for human C5 has been obtained and it includes the C terminal 262 amino acids of the β chain, the C5a fragment split from the α chain on activation, and the N terminal 98 residues of the α′ chain (Lundwall *et al.*, 1985).

The strong sequence homology between C3, C4, and C5 is shared with α_2-macroglobulin. This is a protease inhibitor present in blood, also synthesized as a single chain polypeptide of about 180,000 M_r and for which a complete amino acid sequence has been obtained (Sottrup-Jensen *et al.*, 1983). Another protein showing sequence homology with α_2-macroglobulin and presumably of the same family is the pregnancy zone protein (Sottrup-Jensen *et al.*, 1984). It is present in trace amounts in the plasma of males and nonpregnant females, but rises to about 1 g/liter in late pregnancy and its function is not yet clear.

VII. C9

The lytic complex is formed by self-assembly of the late components after the activation of C5 (Fig. 2). The C5,6,7,8 complex inserts into the membrane and appears to act as a specific binding site for the polymerization of C9 but isolated C9 will also polymerize though more slowly (Podack and Tschopp, 1982). This ability of C9 alone to form a polymeric integral membrane protein suggests that it will contain hydrophobic sections. The primary amino acid sequence has been obtained from a human cDNA clone (DiScipio *et al.*, 1984) and it shows the C9 peptide chain to contain 537 amino acids, the C terminal half of which are generally hydrophobic in character. cDNA clones of C9 have also been obtained from the human liver cDNA expression library in which positive clones were identified by specific antibodies (Stanley and Luzio, 1984; Stanley *et al.*, 1985).

VIII. C4b-Binding Protein (C4bp)

C4bp has a molecular weight of about 550,000 and is formed from 7 or 8 identical peptide chains of 70,000 M_r. A cDNA clone from a human liver library (Chung *et al.*, 1985a) together with amino acid sequencing (Chung *et al.*, 1985b) have given the complete amino acid sequence of 549 residues. It

is likely that polysaccharides are attached at positions 173, 458, and 500. An unusual feature is that the N-terminal 491 amino acids are divided into 8 homologous regions in which there are highly conserved residues such as 4 half-cystines, 1 tryptophane, and 1 proline. Electron microscopic studies showed C4bp as a spider-like structure with a head and seven flexible tentacles (Dahlbäck *et al.*, 1983). It is possible that the N-terminal repetitive sequences form the tentacles and the C-terminal ends are joined to form the heads.

C4bp is able to bind to C4b displacing C2a in the C3 convertase, C4b2a, and it also serves as a cofactor in the subsequent hydrolysis of C4b by Factor I. It is of interest therefore that Factor B which interacts with the homologous protein C3b has 3 N-terminal regions of internal homology of about 60 residues (Morley and Campbell, 1984) and that these are also homologous to the 8 N-terminal homologous regions of C4bp. C2 similarly has 3 homologous regions comparable to those of Factor B and of C4bp (D. R. Bentley, unpublished). This suggests that there is a structure on the surface of C4b which can interact with this 60 residue repeating sequence found on C4bp and on C2. Similar structures on C3b would then be expected to interact with the Factor B internal homology region. It would be expected that the amino acid sequence of Factor H would show similar homologous regions as Factor H has the equivalent role to C4bp and this has proved to be correct (Kristensen *et al.*, 1985). Factor H will displace Factor B from the alternative pathway C3 convertase C3bBb and is a cofactor for the hydrolysis of C3b by Factor I. CR1, the C3 receptor, binds C3b and also acts as cofactor to Factor I in C3b digestion so if this internal repeating homology unit is the site of binding to C3b it would be expected to occur again in the CR1 receptor. The evidence mentioned earlier that the genes coding for CR1, C4bp, and Factor H are linked suggests that they may indeed have homologous structures. It seems likely that the proteins C2, C4bp, and CR1 which bind to C4b will all have homologous repeating units of about 60 amino acids and that Factor B, Factor H, and CRI, which bind to the closely related C3b, will have similar homologous repeating units. If correct, this is a remarkable conservation of genetic material coding for parts of 5 proteins with a closely related binding region but otherwise with quite different biological activity. C2 and Factor B are proteolytic zymogens, C4bp and Factor H are cofactors for the protease Factor I, and CR1 is a cell surface receptor as well as cofactor for Factor I. The C2 and Factor B genes are not linked to the C4bp, Factor H, and CR1 genes. If the homologous regions of these proteins have a common genetic origin, an unusual mechanism must have been involved in their evolution. It is not, however, unique. A 40 amino acid residue repeating sequence has been observed in low-density lipoprotein (LDL) receptor which is homologous to a repeating unit in epidermal growth factor (Südhof *et al.*, 1985a,b)

and it may also occur in Factor IX (Anson *et al.*, 1984) and in two other blood clotting proteins, Factor X and protein C (Doolittle *et al.*, 1984). Further, another repeating sequence in LDL receptor is homologous to a sequence in C9.

Thus there are 3 examples of homologous repeating sequences and, where known, they all correspond with exons. It seems likely that the duplication and switching of exons may have been an important feature of the evolution of many proteins (Gilbert, 1985). It is notable that the exon switching occurs between unlinked as well as linked genes which code for proteins of different biological activity, though perhaps sometimes with partially related functions.

IX. Summary

Rapid progress has been made in establishing linkages and in chromosome allocation of the genes of some 9 complement components. In the MHC, C2, Factor B, and two C4 or C4 related genes have been placed in some detail in both man and mouse. The gene coding for the cytochrome *P*-450 21-hydroxylase has been shown to be duplicated and immediately 3′ to the two C4 genes, though it appears to be functionally and structurally unrelated to the complement components. Thus six genes have been mapped to this region where particular haplotypes are associated with increased susceptibility to a number of diseases, some of which are autoimmune in character.

The complete gene structure of Factor B has been solved in man and rapid progress is being made with the C2 and C4 genes. The structural basis of the polymorphisms of these genes is being established. In C4, the polymorphism is exceptionally complex with varying numbers of loci and probably more than 50 allotypes occurring in man. A structural basis has also been found for the big differences in the biological activity of some of the C4 allotypes in man.

Apart from the genes in the MHC, linkage has been found between the genes coding for C4bp, CR1, and Factor H. Remarkably there are sequence homologies between these proteins and C2 and Factor B, probably related to the ability to bind to one or other of the structurally similar proteins C3b and C4b. The complete cDNA sequences of C3 and C4 in mouse and man have given much information on the many posttranslational modifications of these proteins. A partial structure has been obtained for the C3 gene and the homology shown between C3, C4, C5, α_2-macroglobulin, and pregnancy zone protein.

Although the amount of detailed information in the molecular genetics of complement components is accumulating rapidly, there appears to be a reasonable prospect that linkages and homologies will classify the data into a comprehensible form.

ACKNOWLEDGMENTS

We thank members of the MRC Immunochemistry Unit for their valuable criticism of this article.

MCC is an Investigator of the American Arthritis Foundation.

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ADVANCES IN IMMUNOLOGY, VOL. 38

Molecular Genetics of Human B Cell Neoplasia

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I. Introduction

Since the discovery of the Philadelphia chromosome in the neoplastic cells of patients with chronic myelogenous leukemia (Nowell and Hungerford, 1960), consistent cytogenetic changes have been detected in numerous malignancies of the hematopoietic system (Rowley, 1973, 1983). Many of these chromosomal alterations consist of reciprocal translocations (Rowley, 1983; Yunis, 1983).

While the consistency of such rearrangements in the various leukemias and lymphomas suggested a possible role for these changes in the pathogenesis of human hematopoietic neoplasms, this could not be demonstrated definitely with available techniques.

Recent developments in the identification and characterization of viral genes capable of inducing tumors in experimental animals, however, have been clearly providing major advances in our understanding of the molecular basis of the neoplastic process and of the cellular genes that may be involved in human cancer. The demonstration that chromosomal segments involved in the specific translocations contain the human homologues of viral oncogenes capable of inducing tumors in experimental animals has opened new avenues in our understanding of the role of specific chromosomal alterations in the development of human neoplastic diseases (Dalla Favera *et al.*, 1982a, 1983; Taub *et al.*, 1982; Erikson *et al.*, 1983a).

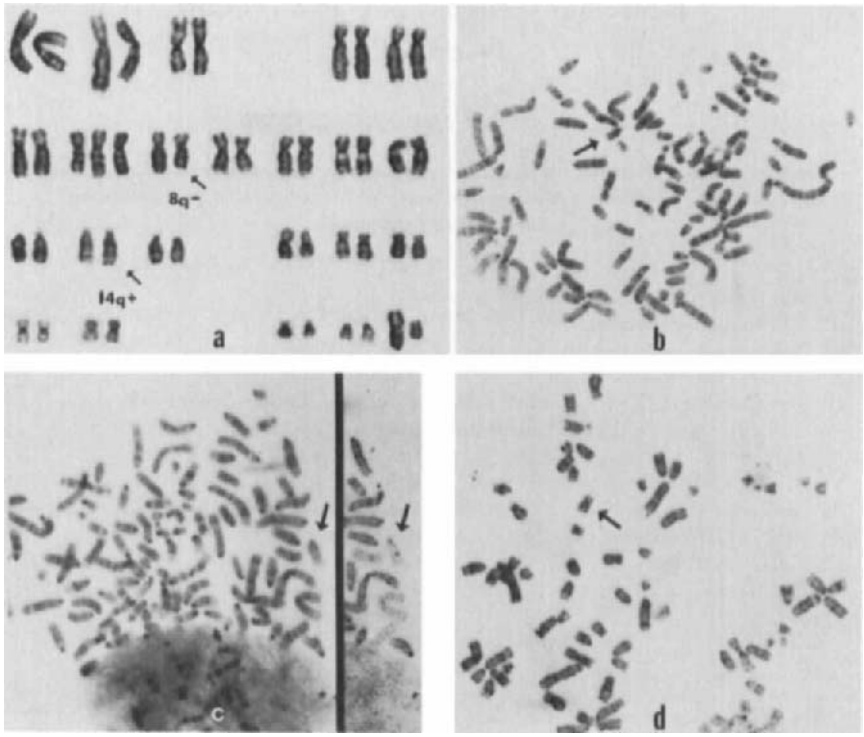

FIG. 1. (a) Karyotype of parental Daudi cell line with t(8;14) (q24;q32) (arrows) and small interstitial deletion in 15q. Trisomy 7 was present in a minority of the cells. (b) Trypsin-Giemsa banded metaphase from hybrid 3E5 Cl 3 containing normal 14 (arrows) and no $14q^+$. (c) Trypsin-Giemsa banded metaphase from hybrid 3F2 with $14q^+$ (arrow) and no normal 14. G-11 staining of the same metaphase (inset) indicates the human origin of $14q^+$. (d) Trypsin-Giemsa banded metaphase from hybrid 1E8 Cl 2 containing human $8q^-$ (arrow) and no 14 or $14q^+$.

II. Chromosomal Translocations in B Cell Neoplasia

Manolov and Manolova (1972) described a consistent chromosome marker in the malignant cells of patients with Burkitt lymphoma, a B cell neoplasm affecting predominantly children. This marker ($14q^+$) derives from a chromosome 14 to which a small segment has been added at the tip of the long arm (Fig. 1). Subsequently, Zech and her associates (1976) found that the $14q^+$ chromosome results from the translocation of the distal end of the long (q) arm of chromosome 8 (band q24–>qter) to the distal end of the long (q) arm of chromosome 14. This translocation was found in cases of Burkitt lymphomas from regions of Africa, in which the tumor is endemic, and from other regions of the world such as Western Europe and America.

Interestingly, the Epstein–Barr virus seemed to have a role in the patho-

genesis of Burkitt lymphoma, since approximately 98% of cases of African Burkitt lymphoma carry the DNA sequences of this virus that can immortalize B cells *in vitro* (Klein, 1981). The great majority (approximately 90%) of Burkitt lymphomas outside Africa, however, do not contain the Epstein–Barr virus genome, suggesting that the virus per se is not essential for the development of this tumor.

Until a few years ago it was thought that the t(8;14) translocation was the hallmark of Burkitt lymphomas. During the last few years, however, several investigators have described variant chromosome translocations in some cases from different countries (Van den Berghe *et al.*, 1979a; Lenoir *et al.*, 1982). These variant translocations involve the same segment of chromosome 8, but instead of translocating to chromosome 14, this segment is translocated to either the long arm of human chromosome 22 (band q11) or to the short arm of chromosome 2 (band p11.2) (Van den Berghe *et al.*, 1979b; Lenoir *et al.*, 1982; Emanuel *et al.*, 1984). Thus, the common denominator of all cases of Burkitt lymphomas is a translocation of the distal end (band q24–>qter) of the long arm of chromosome 8 to either chromosome 14 (band q32), 22 (band q11), or 2 (band p11.2). Approximately 75% of Burkitt lymphomas carry the t(8;14) translocation, 16% carry the t(8;22) translocation and 9% carry the t(2;8) translocation. Somatic cell genetic studies have shown that the three immunoglobulin loci for heavy chains, λ light chains and κ light chains, are located on human chromosomes 14 (Croce *et al.*, 1979), 22 (Erikson *et al.*, 1981), and 2 (Malcolm *et al.*, 1982; McBride *et al.*, 1982), respectively, strongly suggesting a relationship between chromosome translocations and immunoglobulin genes in Burkitt lymphoma (Erikson *et al.*, 1981). The same reciprocal translocation involving human chromosome 8 and chromosome 14 has also been detected in very aggressive human acute lymphocytic leukemias (L3 according to FAB classification) of the B cell type.

Translocations involving human chromosome 14 and different "donor" chromosomes have been observed in other B cell neoplasms of adults. In a fraction of human chronic lymphocytic leukemias of the B cell type, in diffuse large cell and small cell lymphoma, and in a fraction of cases of multiple myeloma the reciprocal chromosome translocation involves human chromosomes 11 (band q13) and 14 (band q32) (Van den Berghe *et al.*, 1984). In follicular lymphomas, the reciprocal chromosomal translocation often involves human chromosomes 14 (band q32) and 18 (band q21) (Yuns, 1983). Thus, it can be concluded that translocations involving the chromosomal regions carrying the immunoglobulin loci are observed quite often in B cell neoplasms of humans.

Similar chromosomal translocations have also been observed in B cell tumors of other mammals. In mineral oil induced myelomas of mice and rats,

chromosomes carrying immunoglobulin genes are commonly involved in translocations (Klein, 1981). In mineral oil-induced multiple myeloma of the mouse the reciprocal translocations involve either chromosome 12 (that carries the heavy chain locus) and chromosome 15, or chromosome 6 (that carries the κ chain locus) and chromosome 15 (Klein, 1981).

In order to determine whether the human immunoglobulin genes are directly involved in the chromosomal rearrangements observed in Burkitt lymphomas, we have used somatic cell hybridization techniques to segregate the relevant human chromosomes on a mouse myeloma chromosome background (Erikson *et al.*, 1982).

As shown in Fig. 1 the human chromosomes involved in the t(8;14) chromosomal translocations segregated in the various hybrid clones, allowing the determination that while the genes for the variable regions of heavy chains are translocated to the involved chromosome 8 ($8q^-$) the genes for the constant regions of heavy chains remain on the involved chromosome 14 ($14q^+$) (Erikson *et al.*, 1982). This could be achieved by Southern blotting analysis of hybrid cell DNAs with nucleic acid probes specific for the variable and the constant regions of heavy chains (Erikson *et al.*, 1982). These results indicate that the chromosome breakpoints in Burkitt lymphoma with the t(8;14) chromosome translocation involve directly the heavy chain locus and that the genes for the variable regions of heavy chains are more distal than the genes for the constant regions on band q32 of chromosome 14 (Erikson *et al.*, 1982). Since hybrids containing the normal human chromosome 14 expressed human heavy chains, while hybrids containing the $14q^+$ did not (Erikson *et al.*, 1982) it can also be concluded that the chromosomal translocation involves the excluded heavy chain locus (Erikson *et al.*, 1982, 1983a).

III. Translocation of the c-*myc* Oncogene in Burkitt Lymphomas with the t(8;14) Chromosome Translocation

By taking advantage of nucleic acid probes specific for the human homologs of retroviral (Dalla Favera *et al.*, 1982b) oncogenes and rodent–human somatic cell hybrids, it has been possible to determine the chromosomal location of several human oncogenes (Dalla Favera *et al.*, 1982a, 1983). Of particular importance was the chromosomal localization of the human homolog of the c-*myc* oncogene, since the *myc* containing avian myelocytomatosis virus can induce B cell lymphomas in chicken (Duesberg, 1979; Hayward *et al.*, 1981). Analysis of the DNA of a panel of somatic cell hybrids between rodent and human cells indicated that the c-*myc* oncogene is located on human chromosome 8 (Dalla Favera *et al.*, 1982a). In order to determine whether the oncogene is located in that small segment of chromosome 8 that is

involved in Burkitt lymphoma with the t(8;14) translocation, somatic cell hybrids carrying the $14q^+$ chromosome in the absence of the normal chromosome 8 and of the $8q^-$ were studied for the presence of the human c-*myc* oncogene. The results of these studies indicated that the c-*myc* oncogene is consistently translocated to the involved human chromosome 14 in Burkitt lymphomas with the t(8;14) translocation (Fig. 2) (Dalla Favera *et al.*, 1982a, 1983; Erikson *et al.*, 1983a).

Restriction enzyme analysis of DNA of Burkitt lymphoma cells using a *myc* DNA probe indicates that in approximately 50% of the cases studied the c-*myc* oncogene is structurally rearranged, while in the remaining 50% it shows a germ line pattern (Dalla Favera *et al.*, 1982a, 1983; Taub *et al.*, 1982). When the c-*myc* gene is rearranged it is recombined in a head-to-head fashion (5′ to 5′) with one of the immunoglobulin constant region genes, predominantly Cu (Fig. 2) (Dalla Favera *et al.*, 1982a, 1983; Taub *et al.*, 1982).

Considerable heterogeneity of breakpoints is observed in Burkitt lymphomas with the t(8;14) chromosome translocation (Croce and Nowell, 1985). On chromosome 14 the breakpoint may occur in the region carrying V_H genes (Erikson *et al.*, 1982), in the joining (J_H) segment (Dalla Favera *et al.*, 1983) and in the switch regions (Taub *et al.*, 1982; Dalla Favera *et al.*, 1983; Showe *et al.*, 1985) of the heavy chain genes (Fig. 3). On chromosome 8 the breakpoint may occur at variable distances from the 5′ end of the c-*myc* gene or it may involve either the first c-*myc* exon or the first c-*myc* intron. As shown in Fig. 3 the c-*myc* gene is formed by three separated exons. The first exon contains termination codons on all three reading frames and therefore represents an untranslated leader sequence (Watt *et al.*, 1983a,b; Leder *et al.*, 1983). The first ATG (methionine) signal for protein synthesis is at the beginning of the second exon and it is followed by an open reading frame encoding for a protein of 439 amino acids (Watt *et al.*, 1983a). The c-*myc* oncogene has also two promoters separated by approximately 160 nucleotides, and the c-*myc* transcripts initiate from two different initiation sites (Fig. 4) (Watt *et al.*, 1983b; ar-Rushdi *et al.*, 1983).

When the c-*myc* gene is decapitated by the chromosomal break leading to the translocation, new cryptic promoters are activated within the c-*myc* first intron and the c-*myc* transcripts initiate from different sites within the first intron (ar-Rushdi *et al.*, 1983) (Fig. 4). As shown by Colby *et al.* (1983), the DNA region in front of the c-*myc* second exon also contains termination codons on all three reading frames and it is noncoding. Therefore, whether the c-*myc* oncogene is rearranged or not the c-*myc* protein is the same. Recently, Rabbitts *et al.* (1984) have reported mutations in the coding exons of the c-*myc* oncogene. Such alterations are not the rule in Burkitt lymphomas and are observed in only a minority of cases, indicating that alterations

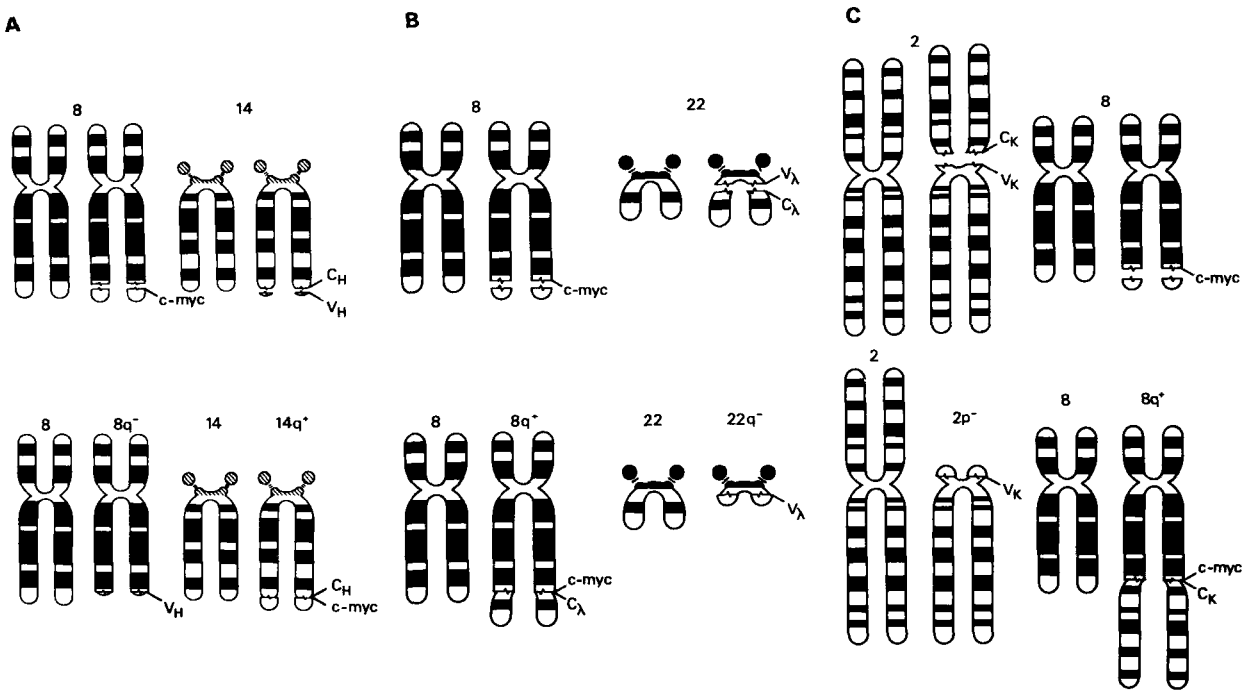

FIG. 2. Diagram of the t(8;14) (A), t(8;22) (B), and t(2;8) (C) chromosome translocations observed in Burkitt's lymphoma.

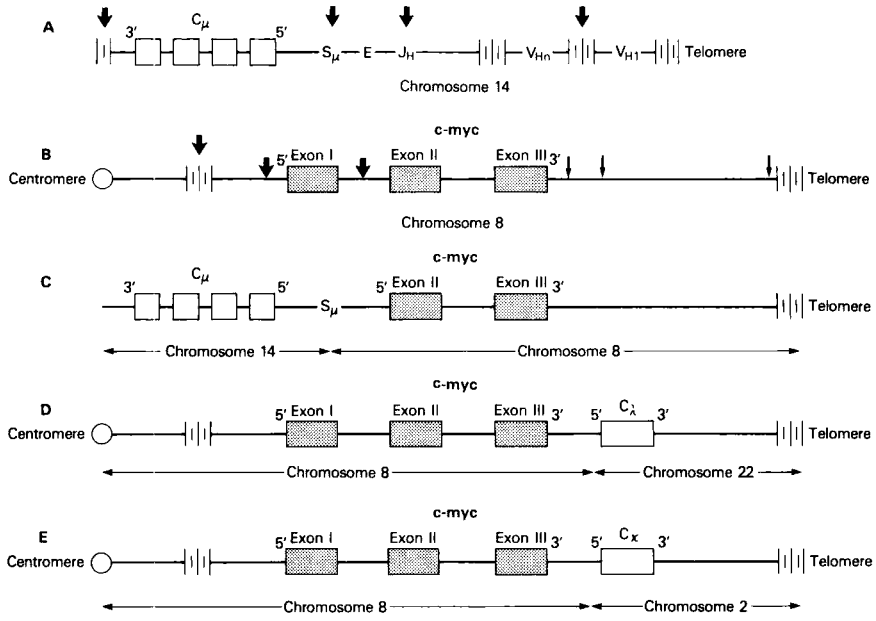

FIG. 3. DNA rearrangements in Burkitt lymphoma. (A) In Burkitt lymphomas with the t(8;14) translocation the chromosomal breakpoints within the heavy chain locus may occur in the region carrying V_H genes, in the region between J_H and V_H, in the heavy chain joining segment (J_H), in a switch region, and may involve a different heavy chain gene such as Cμ, Cγ, and Cα. The arrows indicate possible sites for chromosomal break. The position of the heavy chain enhancer (E) is indicated. (B) In Burkitt lymphomas with the t(8;14) translocations the chromosomal breakpoints on chromosome 8 are 5′ of the two coding exons (II and III) of the c-*myc* oncogene (thick arrows). In some cases the c-*myc* oncogene is decapitated by the chromosomal break and the first exon of the gene remains on the $8q^-$ chromosome, while the coding exons translocate to chromosome 14 (see C). In Burkitt lymphomas with the variant t(8;22) and t(2;8) translocations the breakpoints are distal to the c-*myc* oncogene (thin arrows). (C) Example of a Burkitt lymphoma with the t(8;14) translocation and a rearranged c-*myc* gene. The Cμ gene and the c-*myc* oncogene are involved in a head-to-head rearrangement. The transcription of the c-*myc* and of the Cμ genes are in opposite directions (5′–>3′). (D) In Burkitt lymphomas with the t(8;22) translocation the c-*myc* oncogene remains on chromosome 8, while the λ locus translocates to a chromosomal region 3′ (distal) to the c-*myc* oncogene (see B). (E) In Burkitt lymphomas with the t(2;8) translocation the c-*myc* oncogene also remains on chromosome 8, while the κ locus translocates to a chromosomal region 3′ (distal) to the c-*myc* oncogene (see B).

of the c-*myc* coding sequences are not necessary for malignant transformation.

Figures 2 and 3 summarize the findings concerning the genetic changes in Burkitt lymphomas with the t(8;14) translocation. Note that the chromosomal breakpoint on human chromosome 8 is always 5′ (proximal) to the c-*myc* coding exons (exons II and III) (Figs. 2 and 3).

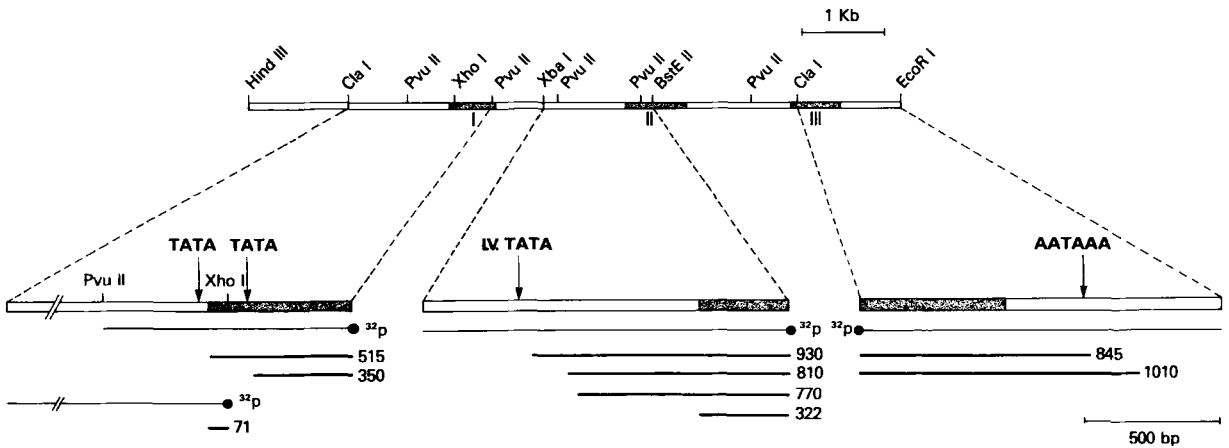

FIG. 4. Schematic representation of DNA probes used for S1 nuclease analysis. The structure of the human c-*myc* genomic DNA is shown schematically according to previous data. A pBR322 subclone, pMyc41.HE carrying the 8.3 kb *Hin*dIII–*Eco*RI DNA fragment shown in this figure, was used to prepare various S1 probes. A double-stranded 1.3 kb *Cla*I–*Xho*I fragment, 5′^{32}P labeled at the *Xho*I site within the first exon, and a 0.8 kb fragment 5′ ^{32}P labeled at the *Pvu*II site, were used to analyze the initiation sites. The probe used for S1 mapping analysis to detect the novel initiation sites or cryptic splicing sites within the intervening sequences between the first and second exons was a double-stranded 1.4 kb *Xba*I–*Bst*EII fragment, 5′ ^{32}P labeled at the *Bst*EII site within second exon. The probe used for S1 nuclease analysis of the 3′ end of the c-*myc* messages was a 1.4 kb DNA fragment, *Cla*I–*Eco*RI, labeled with ^{32}P at the 3′ end. The location and size of S1 nuclease-resistant DNA products are shown together by the solid bars in the diagram. The approximate location of the authentic TATA boxes found by us and another "TATA box-like" sequence (I.V. TATA) found within the first intron are indicated. The location of the recognition signal sequence (AATAAA) for polyadenylation found by Colby *et al.* (1983) is indicated.

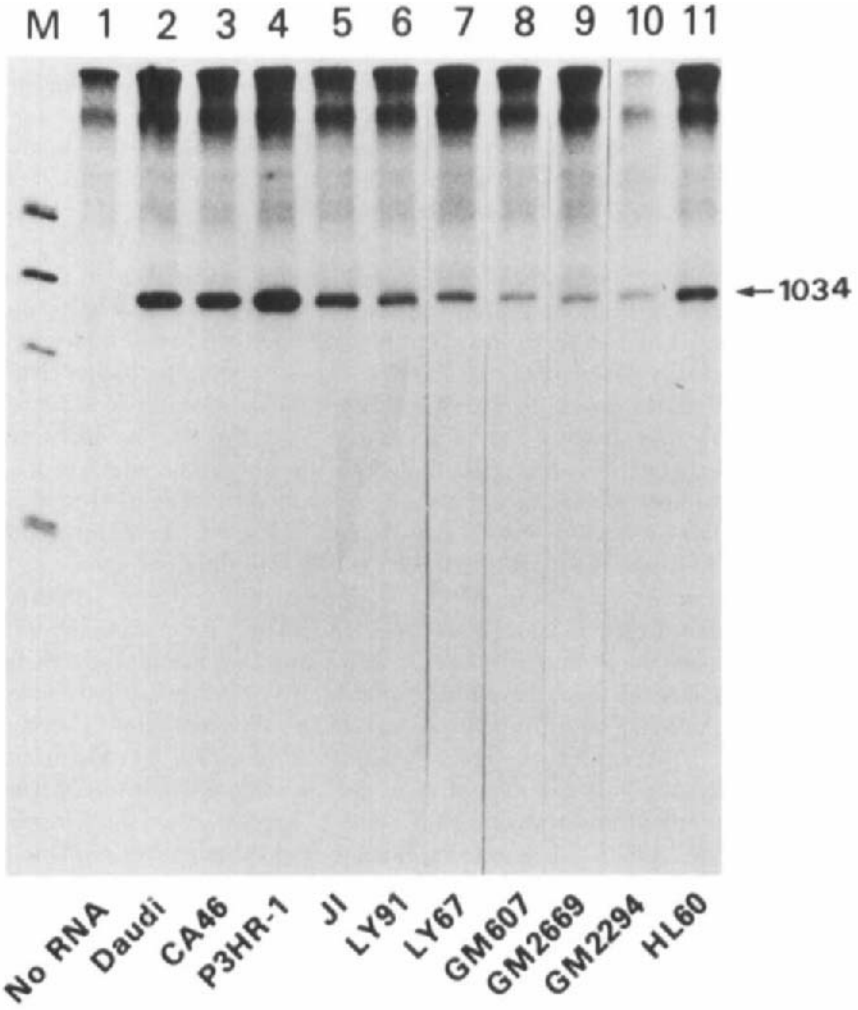

FIG. 5. Detection of the transcripts produced from the c-*myc* gene in various Burkitt lymphoma cells and human lymphoblastoid cell lines by S1 nuclease analysis. The probe, cleaved with *Bcl*I and 5′ ^{32}P-end-labeled pRyc 7.4 plasmid, was heat denatured, hybridized in 80% formamide to 20 μg of cytoplasmic RNA at 55°C, digested with S1 nuclease, and analyzed by electrophoresis in a 7 *M* urea 4% polyacrylamide gel. Lanes 2–7, RNAs were from Burkitt lymphoma cells Daudi, CA46, P3HR-1, JI, LY91, and LY67, respectively. Lanes 8–10, RNAs from GM607, GM2669, and GM2294 lymphoblastoid cells. Lane 11, RNA from promyelocytic leukemia cell line HL-60. Lane M, size marker: ϕX174 digested with *Hae*III and 5′ ^{32}P-end labeled. The size of the protected DNA fragment by using the pRyc 7.4 probe was 1034 nucleotides.

IV. Deregulation of the Translocated c-*myc* Oncogene in Burkitt Lymphoma

If the *myc* gene product is the same in Burkitt lymphoma cells and in human normal B cells, what are the consequences of the t(8;14) chromosome translocation leading to malignant transformation? By examining the steady state levels of human *myc* transcripts in different Burkitt lymphoma cell lines, we found that these levels are quite variable from case to case but are generally elevated (Erikson *et al.*, 1983a; Nishikura *et al.*, 1983). As shown in Fig. 5 the c-*myc* DNA levels in Burkitt lymphomas are similar to those observed in the HL-60 human promyelocytic leukemia cell line where the c-*myc* gene is present in approximately 40 copies per cell because of gene amplification (Dalla Favera *et al.*, 1982a; Nowell *et al.*, 1983) and higher than in three EBV transformed human lymphoblastoid cell lines which were nontumorigenic in nude mice (Nishikura *et al.*, 1983) (Fig. 5). The difficulty with the experiment just described is that we do not know what are the normal counterparts of the Burkitt lymphoma cells that were used in this study. Therefore, it was impossible to compare the c-*myc* RNA levels in malignant and normal cells at the same stage of B cell differentiation.

In order to overcome this problem, we decided to determine whether there is a difference in the levels of transcripts of the normal versus the translocated c-*myc* oncogene in the same cells. Thus, we introduced either the normal c-*myc* gene on chromosome 8 or the translocated c-*myc* oncogene on the $14q^+$ chromosome into the same mouse myeloma cells by using somatic cell hybridization techniques (Nishikura *et al.*, 1983). As shown in Fig. 6 the normal c-*myc* gene is not expressed in a mouse myeloma background, while the translocated c-*myc* gene is expressed at high levels (Nishikura *et al.*, 1983). These results indicate that there is a fundamental difference between the expression of the normal and the translocated c-*myc* gene: the normal c-*myc* gene is transcriptionally silent in the background of terminally differentiated B cells, but the translocated c-*myc* gene is expressed constitutively at elevated levels (Nishikura *et al.*, 1983; Croce *et al.*, 1983).

To determine whether the normal c-*myc* oncogene is capable of responding to normal transcriptional regulation in a terminally differentiated B cell background we have hybridized human lymphoblastoid cells in which the c-*myc* oncogene is in its germ line configuration on a normal chromosome 8 and is expressed at moderately elevated levels with mouse myeloma cells. The introduction of the active c-*myc* oncogene derived from a lymphoblastoid cell into a mouse myeloma cell results in its repression (Nishikura *et al.*, 1983). Thus we conclude that while the normal c-*myc* gene is capable of responding to normal transcriptional control in a differentiated B cell, the translocated c-*myc* gene fails to respond to these normal control mechanisms

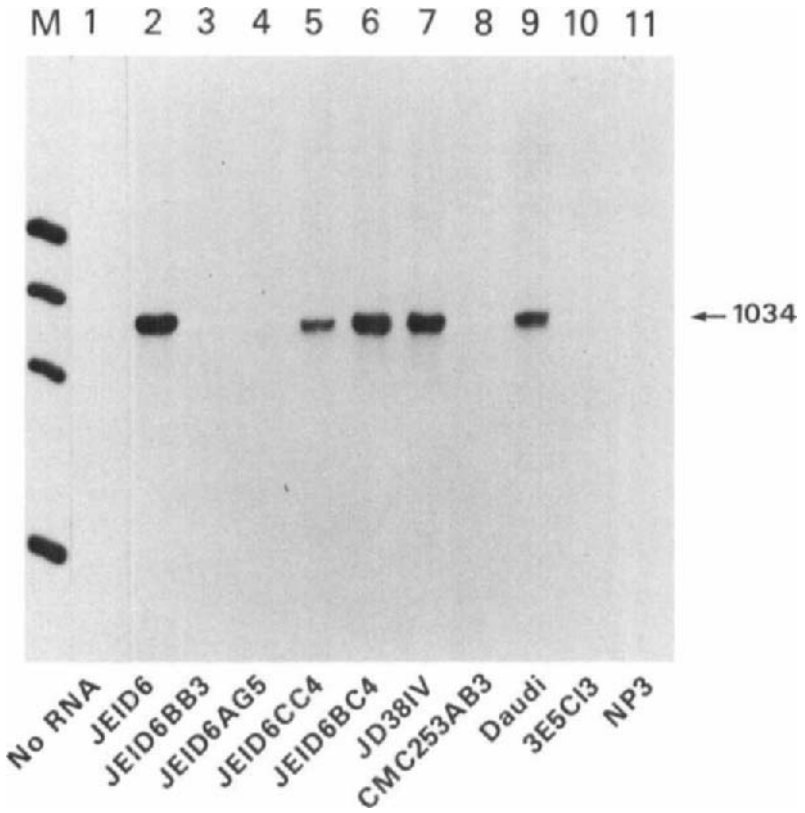

FIG. 6. Expression of the c-*myc* oncogene on the normal chromosome 8 and on the $14q^+$ in hybrids with mouse myeloma. An S1 nuclease protection assay was used to quantitate the expression of the human c-*myc* transcripts in the hybrids. By this method a human c-*myc* cDNA clone can be protected only by human *myc* RNA and not by mouse *myc* RNA. The NP3 mouse myeloma parental cells express high levels of mouse *myc* transcripts (Nishikura *et al.*, 1983). As shown in lane 11, the RNA derived from NP3 does not protect the human *myc* cDNA clone. On the contrary we detect the expression (the protected fragment is 1034 nucleotides in length) of high levels of human *myc* transcripts in two lymphomas, JD38IV and Daudi, carrying the t(8;14) translocation (lanes 7 and 9). In lane 2 is the result of the protection experiment using the RNA of an NP3 × Burkitt hybrid (JE1D6) that contains both the normal chromosome 8 and the $14q^+$. As shown in lane 2 high levels of human *myc* transcripts are expressed in this hybrid. The hybrid JE1D6 was subcloned and hybrid subclones carrying chromosome 8 but not $14q^+$ (JE1D6BB3 and JE1D6AG5) (lanes 3 and 4) and hybrid subclones carrying chromosome $14q^+$ but not the normal 8 (JE1D6CC4 and JE1D6BC4) were analyzed. As shown in Fig. 6, the two hybrid subclones with only chromosome 6 did not express *myc* transcripts (lanes 3–4), while the two hybrid subclones with the $14q^+$ chromosome expressed high levels of human *myc* transcripts (lanes 5 and 6). In lane 8 is a hybrid between JD38IV and NP3 cells which has retained the normal chromosome 8 and lost the $14q^+$ chromosome. In lane 10 is a hybrid between NP3 and Daudi cells which has retained the normal chromosome 8 and lost the $14q^+$ chromosome.

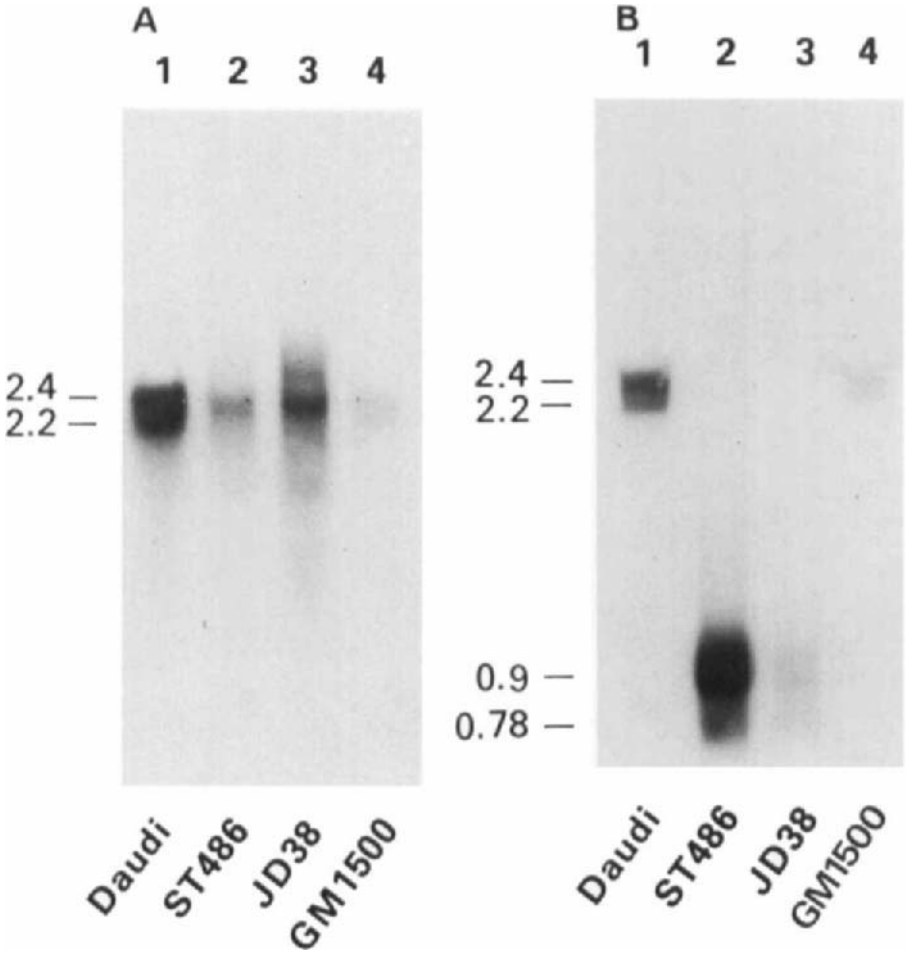

FIG. 7. (A,B) Northern blotting analysis of RNA from three Burkitt's lymphoma cell lines and from an Epstein–Barr virus-transformed lymphoblastoid cell line (GM1500). Polyadenylated RNA was extracted, and 5 μg of RNA was added to each lane of 1.4% agarose gel. After agarose gel electrophoresis and transfer to nitrocellulose filters, the RNA was hybridized (A) with the Ryc 7.4 probe specific for the two coding exons and (B) with the 5′ exon probe. Lanes 1, 2, and 3 show the RNA from the lymphoma cell lines Daudi, ST486 and JD38 IV, respectively. Lane 4 shows the RNA from GM1500 lymphoblastoid cells. All cell lines showed the 2.4 to 2.2 kb c-*myc* transcripts with the Ryc 7.4 probe, whereas the 2.4 to 2.2 kb c-*myc* transcripts were detected only in Daudi and GM1500 cells with the 5′ exon probe. We detected 0.9 to 0.7 transcripts hybridizing with the 5′ exon probe in the two lymphoma cell lines with a rearranged c-*myc* oncogene (ST486 and JD38 IV).

which suppress c-*myc* transcription in the context of a terminally differentiated B cell (Nishikura *et al.*, 1983).

Analysis of Burkitt lymphomas carrying a decapitated c-*myc* oncogene translocated to chromosome 14 also indicates that while the translocated c-*myc* gene is expressed at elevated levels, the normal c-*myc* gene is transcriptionally silent in the neoplastic B cells (ar-Rushdi *et al.*, 1983). As shown in Fig. 7A and B hybridization of RNA derived from two lymphomas carrying the t(8;14) chromosome translocation and a decapitated *myc* with a first exon probe indicates that they do not express normal *myc* transcripts (lanes 2 and 3). On the contrary the first exon probes detect 2.2–2.4 c-*myc* transcripts in Daudi Burkitt lymphoma cells in which the translocated *myc* is not decapitated (Dalla Favera *et al.*, 1982a) (lane 1) and also in human lymphoblastoid cells (lane 4) (ar-Rushdi *et al.*, 1983). Expression of the normal c-*myc* oncogene has been detected in few Burkitt lymphoma cell lines (Rabbitts *et al.*, 1984), but the levels of transcripts of the uninvolved c-*myc* gene are much lower than those of the translocated c-*myc* gene (Croce *et al.*, 1985). Rabbitts *et al.* (1984) have speculated that the c-*myc* product is capable of autoregulating c-*myc* transcription and that the lack of expression of the normal c-*myc* allele is due to such autoregulation. This interpretation, however, is not consistent with the observation that in somatic cell hybrids between Daudi cells and human lymphoblastoid cells, both the translocated and the normal c-*myc* genes are transcribed (Croce *et al.*, 1985).

Interestingly, Adams *et al.* (1983) have also shown the lack of expression of the normal c-*myc* gene in mouse plasmacytomas. It was shown previously that such neoplastic B cells have a translocation between chromosomes 12 and 15 (Klein, 1981) where mouse chromosome 12 carries the heavy chain locus and mouse chromosome 15 carries the c-*myc* oncogene (Taub *et al.*, 1982; Marcu *et al.*, 1983).

V. Molecular Genetics of the Variant Chromosome Translocations in Burkitt Lymphoma

By analyzing somatic cell hybrids between mouse myeloma cells and Burkitt lymphoma cells carrying either that t(8;22) or the t(2;8) chromosome translocation, it has been possible to follow the segregation of the relevant human chromosomes and to understand the genetic rearrangements occurring in Burkitt lymphoma cells with the variant chromosomal translocations. As shown in Fig. 2, we have shown that in Burkitt lymphomas with the t(8;22) translocation the c-*myc* oncogene is not translocated but remains on chromosome 8 in its germ line configuration (Croce *et al.*, 1983). Interestingly, the excluded lambda locus translocates to a region 3′ (distal) to the c-*myc* oncogene (Croce *et al.*, 1983). The breakpoint may occur a few hun-

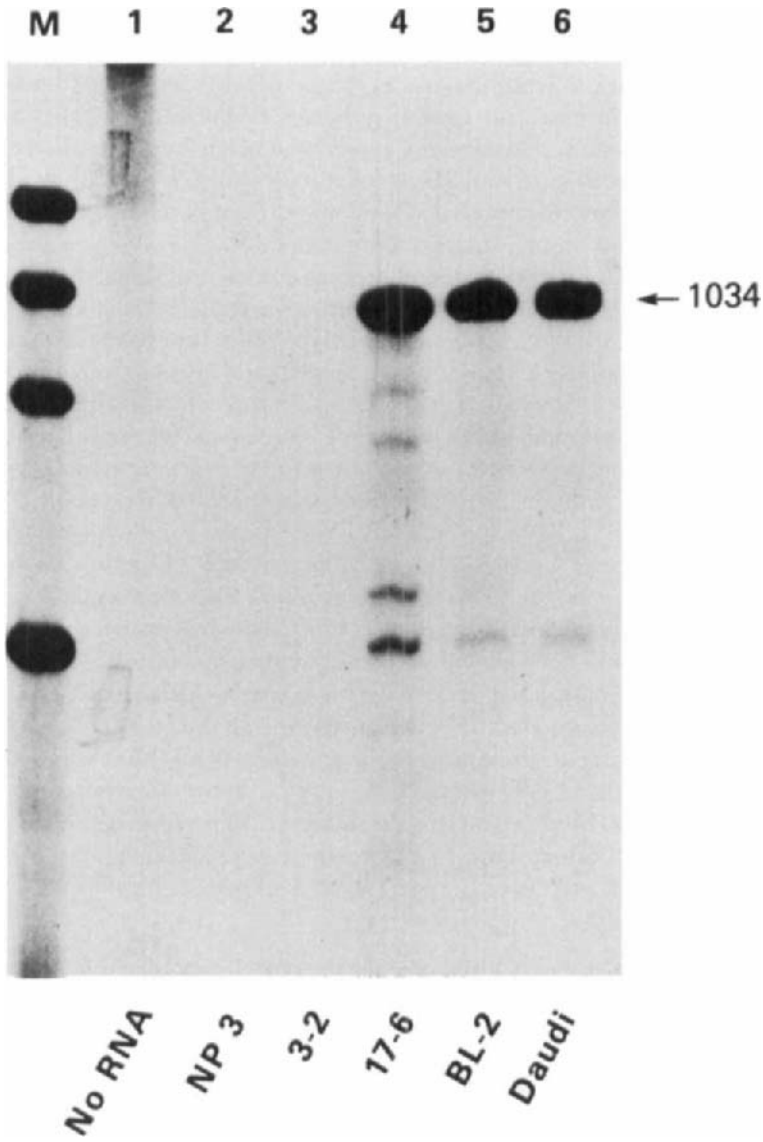

FIG. 8. S1 nuclease protection experiment using a human c-*myc* cDNA probe (Ryc 7.4) specific for the second and third exon of the c-*myc* gene. The human *myc* RNA protects a DNA fragment 1034 nucleotides long. Hybrid BL 17-6, which carries the $8q^+$ chromosome, expresses high levels of c-*myc* transcripts (lane 4). On the other hand hybrid BL 3-2, which contains the normal 8, the normal 22, and the normal $22q^-$ chromosomes, does not express human *myc* transcript (lane 3). The parental BL2 Burkitt Lymphoma cells and Daudi Burkitt lymphoma cells that carry a t(8;14) chromosome translocation express high levels of human c-*myc* transcripts (lanes 5 and 6, respectively). Lane M, marker DNAs.

dred nucleotides or a few kilobases as in BL2 cells (Croce *et al.*, 1983; Erikson and Croce, 1985), or more than 30–40 kb from the 3′ end of the c-*myc* oncogene (Lee and Croce, 1985). Analysis of somatic cell hybrids between mouse myeloma cells and BL2 Burkitt lymphoma cells carrying the t(8;22) translocation indicates that the involved c-*myc* oncogene on the $8q^+$ chromosome is expressed at elevated levels in the hybrids, while the c-*myc* oncogene on the normal chromsome 8 is transcriptionally inactive (Croce *et al.*, 1983) (Fig. 8). Therefore, these results indicate that the c-*myc* oncogene, which is involved in the reciprocal chromosome exchange, is deregulated even if it is not translocated and even if the involved immunoglobulin locus is distal to the activated oncogene (Croce *et al.*, 1983). These results also indicate that the orientation of the λ locus is opposite to the orientation of the heavy chain locus in man (Croce *et al.*, 1983; Emanuel *et al.*, 1985).

We have also examined Burkitt lymphoma cells carrying the t(2;8) chromosome translocation by the somatic cell genetic approach and found that in this case also the chromosomal breakpoint occurs 3′ (distal) to the c-*myc* oncogene, and the light chain locus (κ) translocates to a region that is 3′ (distal) to the c-*myc* oncogene (Erikson *et al.*, 1983b). In Fig. 2 we summarize the different genetic exchanges occurring in Burkitt lymphomas with the t(8;14), t(8;22), and t(2;8) chromosome translocations.

Translocation of the κ locus to a region 3′ to the c-*myc* oncogene also results in the transcriptional deregulation of the involved c-*myc* oncogene (Erikson *et al.*, 1983b). All hybrids between mouse myeloma cells and Burkitt lymphoma cells with the t(2;8) translocation that carry the $8q^+$ chromosome express elevated levels of c-*myc* transcripts, while the hybrids containing the normal chromosome 8 do not express the human c-*myc* gene (Erikson *et al.*, 1983).

In view of these results concerning the regulation of c-*myc* expression in somatic cell hybrids, it can be concluded that the common feature in Burkitt lymphoma is a transcriptional activation of the c-*myc* oncogene involved in the reciprocal chromosomal translocations, leading to its constitutive expression at elevated levels (Nishikura *et al.*, 1983; Croce *et al.*, 1983; Erikson *et al.*, 1983b). It may be inferred that while the c-*myc* gene should be either silent or expressed at very low levels in the normal counterparts of Burkitt lymphoma cells, the close proximity of the involved c-*myc* gene to an immunoglobulin locus results in its transcriptional activation and constitutive expression.

VI. Mechanisms of c-*myc* Activation

Why is the c-*myc* gene that is involved in one of the three different chromosomal translocations deregulated in Burkitt lymphoma? One possibility is that the involved c-*myc* gene is activated by genetic elements

within the three immunoglobulin loci capable of activating gene transcription *in cis* (Croce *et al.*, 1983, 1984, 1985). A different possibility has been proposed by Leder *et al.* (1983), who suggested that the involved c-*myc* oncogene is deregulated because of decapitation or alterations of its 5′ exon or of a region 5′ to the c-*myc* oncogene, with resultant failure to bind a repressor and subsequent transcriptional activation.

In order to determine the mechanisms of c-*myc* activation in Burkitt lymphoma, at first we have asked the question of whether such activation by chromosomal translocation is B cell specific. Results mentioned in the preceding section indicate that the involved c-*myc* gene is expressed in hybrids with mouse myeloma cells. Similarly human immunoglobulin genes are expressed in hybrids between mouse and human B cells (Croce *et al.*, 1979; Erikson *et al.*, 1981; Erikson and Croce, 1982). We have also hybridized Burkitt lymphoma cells carrying the t(8;14) chromosome translocation in which the translocated c-*myc* oncogene has been decapitated of its 5′ exon with different mouse fibroblasts (Nishikura *et al.*, 1984). The hybrids were then studied for the presence of the translocated c-*myc* oncogene and of the productively rearranged μ heavy chain gene. The hybrids containing both the translocated c-*myc* oncogene and the productively rearranged μ gene failed to express both genes. This result indicates that mouse fibroblasts are incapable of transcribing immunoglobulin genes and a translocated c-*myc* oncogene (Nishikura *et al.* 1983, 1984). Since the hybrids contained a decapitated c-*myc* oncogene, it seems quite unlikely that the model proposed by Leder *et al.* (1983) concerning the mechanism of c-*myc* deregulation is correct. If decapitation of the first exon resulted in the failure of the gene to respond to a repressor leading to its deregulation, the decapitated c-*myc* gene should have been expressed in fibroblast hybrids and this was not the case (Nishikura *et al.*, 1984).

To determine whether the activation of the involved c-*myc* oncogene depends on the stage of differentiation of the B cells harboring the translocation chromosome carrying the activated c-*myc* oncogene, we have also hybridized Burkitt lymphoma cells with Epstein–Barr virus (EBV) transformed human lymphoblastoid cells (Croce *et al.*, 1984, 1985). Interestingly we found that such hybrids are phenotypically similar to the lymphoblastoid parental cells, indicating that the lymphoblastoid phenotype is dominant in somatic cell hybrids between these cells (Croce *et al.*, 1984, 1985). Having thus introduced a translocated and activated c-*myc* gene into human lymphoblastoid cells, we carried out S1 nuclease analysis of hybrids containing both the germ line and the translocated c-*myc* oncogene. The results indicated that the translocated c-*myc* gene is not expressed in a lymphoblastoid background (Croce *et al.*, 1984). In the two Burkitt lymphoma cell lines (ST486 and CA46) that we have used for hybridization with human lymphoblastoid cells the decapitated and translocated c-*myc* gene was recombined

with the μ switch region (Sμ) in the case of ST486 cells and with the alpha switch region (Sα) in the case of CA46 cells (Showe *et al.*, 1985). Therefore, in these cases the enhancer located between Sμ and the joining (J_H) segment of the heavy chain gene was translocated to the $8q^-$ chromosome and was not in front of the translocated c-*myc* gene (see Fig. 2C). Since we did not observe translocated c-*myc* activation in the lymphoblastoid hybrids although the translocated c-*myc* oncogene is expressed in the parental human lymphoma cells and in their hybrids with plasma cells, we concluded that the translocated c-*myc* gene is activated by cis-acting genetic elements within the heavy chain locus that are capable of activating transcription in plasma cells and Burkitt lymphoma cells, but not in lymphoblastoid cells (Croce *et al.*, 1984). A corollary of this is that in order to be expressed, the translocated c-*myc* gene must interact with B cell specific transacting factors that are expressed in plasma cells and Burkitt lymphoma cells but not in lymphoblastoid cells (Croce *et al.*, 1984). These results are of considerable interest also because they indicate that the expression of an activated oncogene may be either dominant or recessive depending on the differentiated stage of the cells harboring that activated oncogene. Our inference from these results is that the heavy chain locus contains additional, not yet identified, genetic elements capable of activating gene transcription *in cis* in cells at the appropriate stage of differentiation.

In ST486 Burkitt lymphoma cells the breakpoint on chromosome 8 occurred within the first c-*myc* intron and the breakpoint on chromosome 14 occurred within the Sμ heavy chain region, thus the enhancer, normally located between J_H and Sμ, is translocated to the $8q^-$ chromosome in these cells, 3′ (distal) to the cut off c-*myc* 5′ exon in a tail-to-tail rearrangement (3′ to 3′). Interestingly we observed high levels of transcripts of the cut off first *myc* exon on the $8q^-$ chromosome in ST486 cells (Fig. 7B) and in their hybrids with human lymphoblastoid cells (ar-Rushdi *et al.*, 1983; Croce *et al.*, 1984). This result indicates that genetic elements 5′ to Sμ (possibly the enhancer between J_H and Sμ) are capable of activating the transcription of the first *myc* exon in lymphoblastoid cells (Croce *et al.*, 1984). Thus we could predict that a c-*myc* oncogene translocated 5′ to the J_H segment of the heavy chain locus should be expressed in hybrids between Burkitt lymphoma cells and human lymphoblastoid cells. Therefore we hybridized Daudi Burkitt lymphoma cells where the c-*myc* gene is translocated into one of the V_H regions (and therefore is 5′ to the J_H region) (Erikson *et al.*, 1982) with human lymphoblastoid cells. As shown in Table I, these hybrids retained the phenotype of the lymphoblastoid parental cells. Since the first exon of the translocated c-*myc* oncogene is altered in Daudi cells, we could distinguish between the transcripts of the normal and of the translocated c-*myc* oncogene (Fig. 9) (Croce *et al.*, 1985).

As shown in Fig. 9 the hybrids expressed both the normal and the translo-

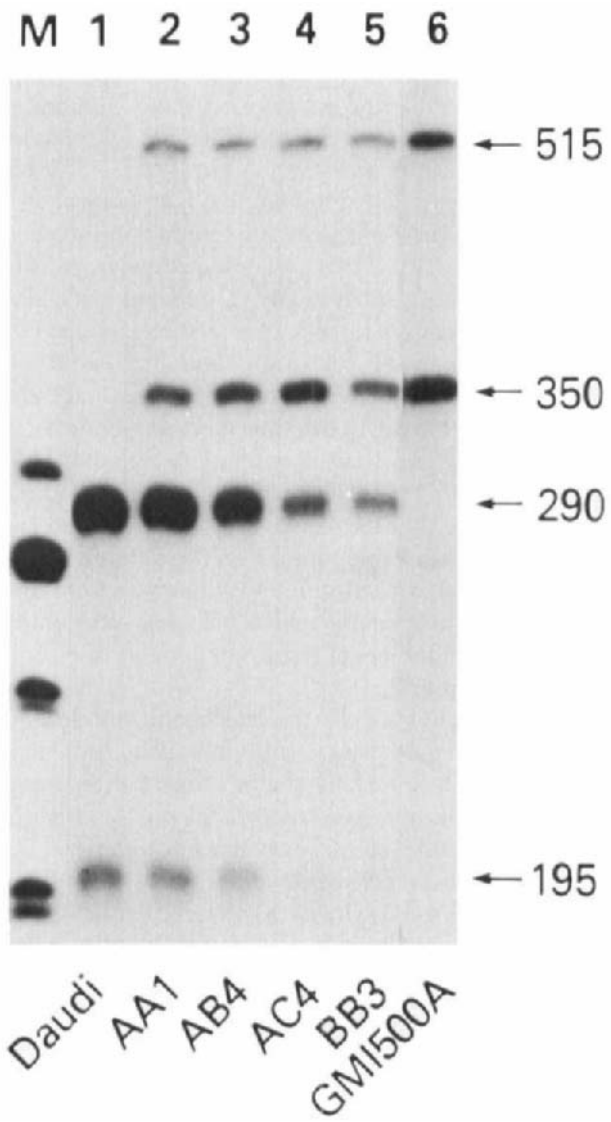

FIG. 9. S1 mapping analysis of c-*myc* mRNAs synthesized in Daudi Burkitt lymphoma and somatic cell hybrids. The RNA was analyzed by the S1 nuclease mapping procedure with modification using a uniformly labeled DNA probe. The *Xma*I–*Pvu*II DNA fragment (610 bp) of the c-*myc* gene containing a portion of the 5′ flanking sequences and the first exon was cloned in M13 and the clone was uniformly labeled with ^{32}P and used as an S1 probe. Cytoplasmic RNAs (20 μg), prepared from various cells by the cesium chloride method, were used for each assay. As previously described, the ^{32}P-labeled DNA probe was heat denatured, hybridized in 80% formamide to cytoplasmic RNAs at 57.5°C for 10 hours, digested with 80 units of nuclease S1, and analyzed by electrophoresis on a 7 *M* urea 4% polyacrylamide gel. M, 5′-^{32}P-labeled ϕX174 *Hae*III digests.

TABLE I

PHENOTYPE OF SOMATIC CELL HYBRIDS BETWEEN DAUDI BURKITT LYMPHOMA AND GM1500-6TG-OUB LYMPHOBLASTOID CELLS

Cells	Immunoglobulin chains[a]				Secretion[b]	Antigen recognized by the B532 antibodies[c]
	μ	γ	κ	κ^1		
Daudi	+	−	−	+	−	−
GM1500-6TG-OUB	−	+	+	−	+	+
AA1	+	+	+	+	+	+
AC4	+	+	+	+	+	+
BB3	+	+	+	+	+	+
AB4	+	+	+	+	+	+

[a] Secreted and cytosol immunoglobulins were labeled with [^{3}H]leucine (100 μCi/ml) and immunoprecipitated by using a rabbit anti-human immunoglobulin antiserum followed by the addition of 50 μl of a 10% suspension of fixed *Staphylococcus aureus* antibodies as described (Croce *et al.*, 1985).

[b] Daudi cells produce membrane bound IgM. Daudi cells express an aberrant κ polypeptide (κ^1) (Croce, *et al.*, 1985). On the contrary GM1500-6TG-OUB secrete IgG (Croce *et al.*, 1985).

[c] Expression of the antigen recognized by the B532 antibodies was studied by indirect immunofluorescence as described (Croce *et al.*, 1985).

cated c-*myc* oncogenes, indicating that a c-*myc* oncogene translocated 5′ to the J_H segment of heavy chain can be expressed in lymphoblastoid cells. This result confirms that genetic elements 5′ to Sμ (possibly the enhancer between J_H and Sμ) are capable of activating translocated c-*myc* gene transcription in Burkitt lymphoma cells, plasma cells, and human lymphoblastoid cells (Croce *et al.*, 1985). Thus it seems that Burkitt lymphoma is a heterogeneous disease in which different genetic elements are involved in the in cis activation of the translocated c-*myc* oncogene. In one case, where the c-*myc* oncogene is translocated to a switch region, the translocated c-*myc* oncogene can be activated only in terminally or near terminally differentiated B cells. In the other case, where the c-*myc* oncogene is translocated to J_H or a region 5′ to J_H, the translocated c-*myc* oncogene can be activated at the different stages of B cell differentiation from pre B cells (Pegoraro *et al.*, 1984) to lymphoblastoid cells, and to plasma cells (Croce *et al.*, 1985). In conclusion, the analysis of c-*myc* activation in Burkitt lymphoma cells using somatic cell hybridization techniques indicates that the hallmark of Burkitt lymphoma is a transcriptional deregulation of the involved c-*myc* gene due to proximity to different genetic elements within the three immunoglobulin loci capable of activating gene transcription in cis over considerable chromosomal distances (Croce *et al.*, 1984, 1985).

These results are of considerable interest not only because they provide insight into the molecular mechanisms of oncogene activation in human

neoplasia, but also because they indicate the existence of different genetic elements within the three immunoglobulin loci capable of activating gene transcription (Croce *et al.*, 1985). Clearly Burkitt lymphoma seems to be a heterogeneous group of diseases in which different genetic elements play an important role in the transcriptional deregulation of the c-*myc* oncogene. In addition it seems quite clear that Burkitt-like translocations are also involved in the pathogenesis of other B cell neoplastic diseases such as acute lymphocytic leukemias of the B cell type (L3 according to the FAB classification). Interestingly, we have recently discovered a human pre B cell leukemia carrying both a t(8;14) and a t(14;18) chromosome translocation characteristic of Burkitt lymphoma and follicular lymphoma, respectively, in which the translocated c-*myc* oncogene is in its germ line configuration and is located more than 15 kb 5′ of the involved J_H segment (Pegoraro *et al.*, 1984). Since expression of the translocated oncogene is elevated in these leukemic cells (ar-Rushdi and Croce, 1985) it can be inferred that activation of the c-*myc* oncogene by chromosomal translocation can occur as early as in pre B cells.

The different rearrangements of the c-*myc* oncogene and of the immunoglobulin loci in malignancies with the t(8;14), t(8;22), and t(2;8) translocations indicate that c-*myc* activation occurs when the c-*myc* oncogene independently of its orientation is located in front (5′) of the involved immunoglobulin locus (Dalla Favera *et al.*, 1982a, 1983; Croce *et al.*, 1983; Erikson *et al.*, 1983a). This finding suggests a polarity in the effect of the immunoglobulin loci in c-*myc* activation. It would be of considerable interest, however, to determine whether there are some cases of Burkitt lymphoma with the t(8;14) chromosomal translocation in which the c-*myc* oncogene remains on chromosome 8 and the enhancer located between J_H and Sμ is translocated 3′ to the involved c-*myc* oncogene. In this case the c-*myc* oncogene and the heavy chain enhancer would be rearranged in a tail-to-tail fashion.

VII. Molecular Genetics of the t(11;14) Chromosome Translocation

Chromosome 14 is involved in reciprocal chromosomal translocations with chromosome 11 in a fraction of chronic lymphocytic leukemias of the B cell type (Nowell *et al.*, 1981; Erikson *et al.*, 1984) in diffuse small cell and large cell lymphomas (Van den Berghe *et al.*, 1979b) and in a sizable fraction (30–40%) of multiple myelomas. Interestingly, while a $14q^+$ chromosome is frequently observed in human multiple myeloma, Burkitt-like translocations are observed quite rarely in this disease or group of diseases. This suggests that mineral oil-induced mouse plasmacytomas may represent only one of the types of plasmacytomas in the mouse. Possibly plasmacytomas carrying other types of translocations may also occur in the rodents.

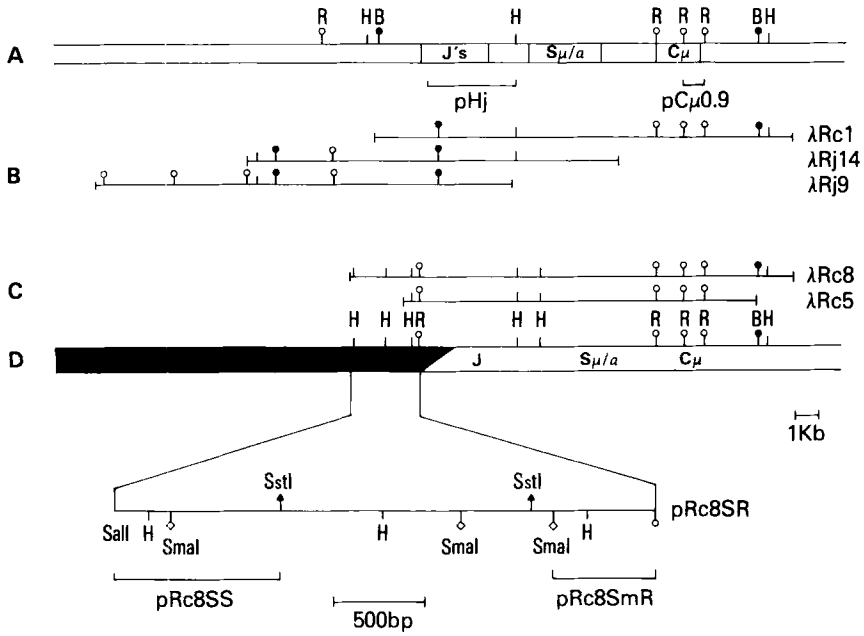

FIG. 10. Restriction maps of the germ line Cμ gene (A) and of the two classes of recombinant clones we have obtained (B and C). Clones shown in B represent the excluded Cμ allele on the $14q^+$ chromosome. H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI. Black box represents the chromosome 11-derived sequences, whereas the white box represents the chromosome 14-derived sequences (D).

Since in human B cell neoplasms with the t(11;14) translocation the chromosomal breakpoints are consistently observed at band q13, we reasoned that the same human locus on chromosome 11 may be involved in the different B cell neoplastic diseases with translocation. By analyzing somatic cell hybrids between mouse cells and chronic lymphocytic leukemia (CLL) cells with the t(11;14) chromosome translocation it was possible to establish that the chromosomal breakpoint on chromosome 14 involves directly the heavy chain locus on the $14q^+$ and that the productively rearranged heavy chain gene is on the normal chromosome 14 (Erikson *et al.*, 1984), similar to the observations in Burkitt lymphoma with the t(8;14) chromosome translocation (Erikson *et al.*, 1982).

We then produced a DNA library of the chronic lymphocytic leukemia DNA in a λ phage vector and screened the recombinant phages with a probe specific for the joining (J_H) segment of the heavy chain locus (Tsujimoto *et al.*, 1984a). As shown in Fig. 10, two classes of recombinant clones were obtained. One class represented the productively rearranged μ heavy chain

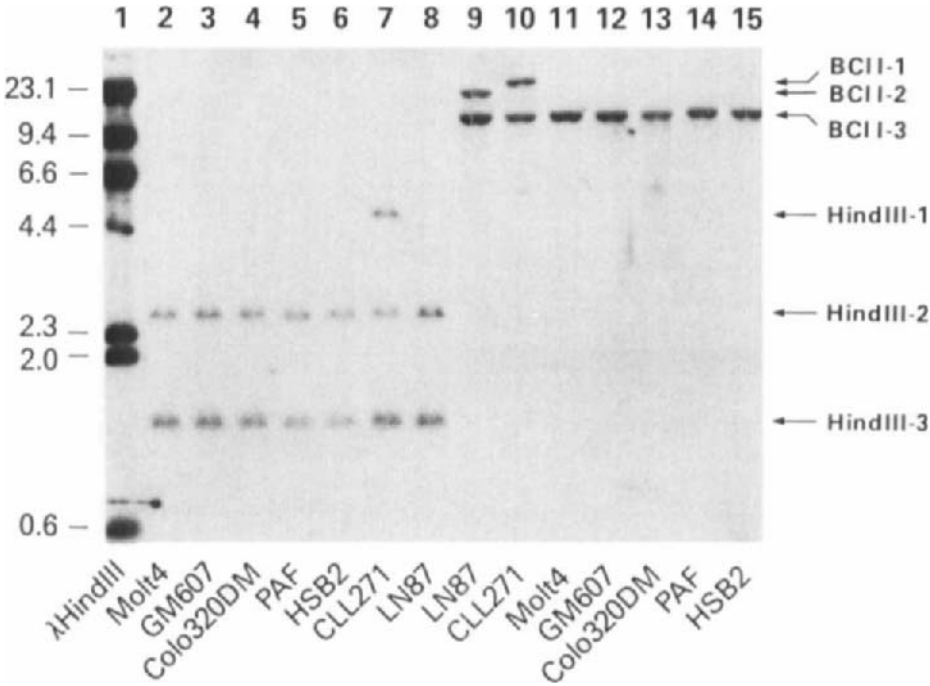

FIG. 11. Southern blot hybridization of CLL 271 and LN87 DNAs with a chromosome 11 specific DNA probe mapping close to the breakpoint of CLL 271 t(11;14). The cellular DNA (5 μg) was digested with *Hin*dIII (lanes 2 to 8) and *Bcl*I (lanes 9 to 15) and fractionated by agarose gel electrophoresis. The Southern blot filter was hybridized with the pRc8SmR probe and finally washed with 0.2 × SSCS at 65°C. The molecular size of the *Hin*dIII cut marker DNA (lane 1) is given in kilobases. Lanes 2 and 11, MOLT 4 (human T-cell line) DNA; lanes 2 and 12, GM607 (human lymphoblastoid cell line) DNA; lanes 7 and 10, CLL 271 (human B cell leukemia) DNA; lanes 8 and 9, LN87 (human diffuse B-cell lymphoma) DNA.

gene on chromosome 14 (Erikson *et al.*, 1984; Tsujimoto *et al.*, 1984b), while the other represented the unproductively rearranged μ gene on the $14q^+$ chromosome (Tsujimoto *et al.*, 1984b). We then subcloned unique DNA sequences 5′ to the rearranged J_H segment of the unproductively rearranged heavy chain locus and found that these DNA probes hybridized to DNA derived from human chromosome 11 but did not hybridize to DNA derived from the other chromosomes, indicating that the second class of recombinant clones contain the joining between chromosomes 11 and 14 on the $14q^+$ chromosome (Tsujimoto *et al.*, 1984a). We then used the chromosome 11 specific probes flanking the breakpoint in the CLL271 cells to detect rearrangements of the homologous DNA sequences in lymphomas and leukemias with the t(11;14) chromosome translocation (Tsujimoto *et al.*, 1984a). As

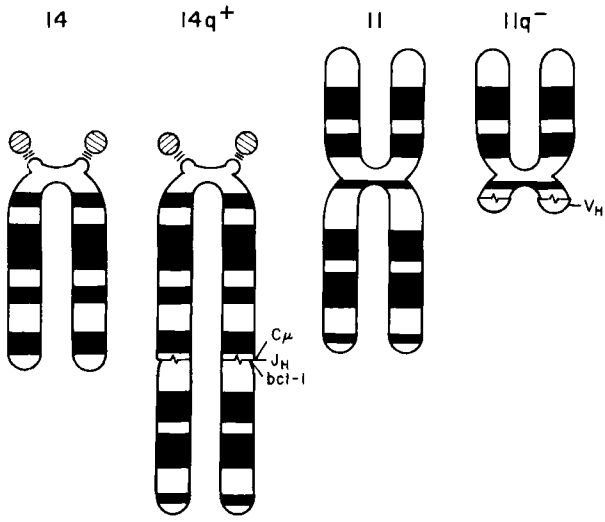

FIG. 12. Diagram of the t(11;14) translocation occurring in human B cell neoplasia.

shown in Fig. 11 we detected rearrangements of the homologous chromosome 11 specific DNA segments in lymphomas and leukemias with the t(11;14) translocation (lanes 9–10) (Tsujimoto *et al.*, 1984a). Additional studies of CLL and of diffuse B cell lymphomas with the same translocation indicate that the breakpoints in different tumors are clustered within a small DNA segment (3.5 kb length) on band q13 of chromosome 11 (Tsujimoto *et al.*, 1984a; Tsujimoto and Croce, 1985). On the basis of these results, we have inferred that on band q13 of chromosome 11 there is a gene, for which we have proposed the name of bcl-1 (B cell lymphoma/leukemia 1) that is activated by its close proximity to the heavy chain locus (Tsujimoto *et al.*, 1984a) (Fig. 12). Since no known homolog of retrovirus oncogenes maps on the long arm of human chromosome 11, we can also infer that the bcl-1 gene is not homologous to any of the presently known retrovirus oncogenes. Interestingly since the chromosome 11 breakpoints in neoplastic B cells with the t(11;14) translocation cluster within a short segment of DNA, it becomes possible to use specific DNA probes to detect the occurrence of a t(11;14) translocation in malignant lymphoid tissues by Southern blotting analysis (Tsujimoto *et al.*, 1984a).

VIII. Molecular Genetics of the t(14;18) Chromosome Translocation

Chromosome 14 is also involved in a reciprocal translocation with chromosome 18 in the great majority of cases of human follicular lymphoma, the

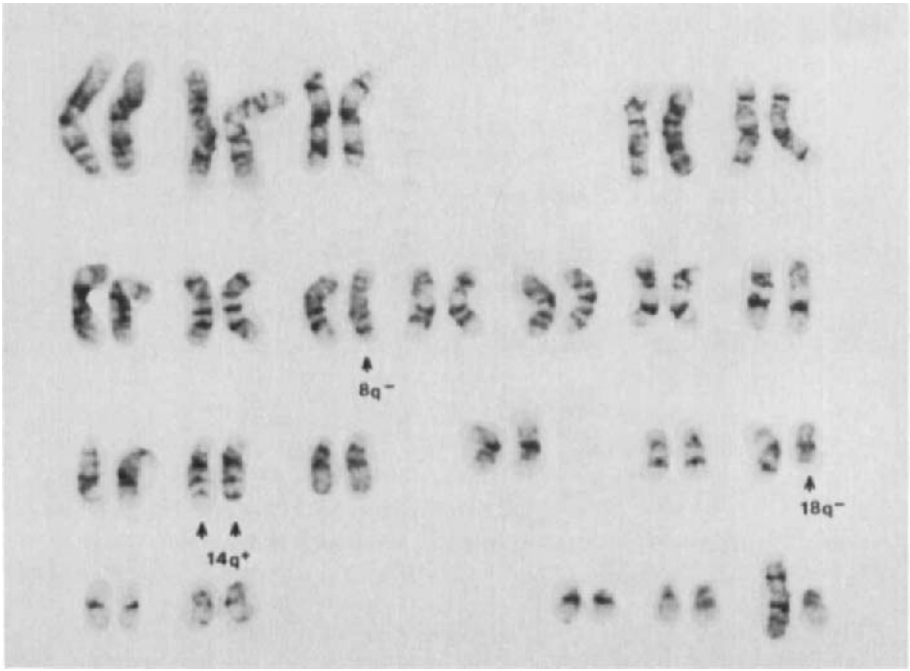

FIG. 13. Karyotype of the 380 cells: 46 XY, t(8;14) (q24;q32). As a result of the two reciprocal chromosomal translocations, the 380 cells have two abnormal chromosomes 14 ($14q^+$), one rearranged chromosome 8 ($8q^-$), and one abnormal chromosome 18 ($18q^-$).

most frequent B cell malignancy in the United States and Western Europe (Yunis, 1983). By taking advantage of an acute B cell leukemia carrying two reciprocal translocations, t(8;14) and t(14;18) (Fig. 13) (Pegoraro *et al.*, 1984), we have been able to clone both chromosomal breakpoints on the two $14q^+$ chromosomes (Fig. 14). As shown in Fig. 14 two classes of recombinant clones were obtained. One class contained the joining between chromosome 8 and 14 on one $14q^+$ chromosome (Fig. 14) (Tsujimoto *et al.*, 1984b). The other class contained the joining between chromosomes 14 and 18 on the other $14q^+$ chromosome (Tsujimoto *et al.*, 1984b). In fact, DNA probes free of repetitive sequences 5′ to the J_H regions of the involved heavy chain loci hybridized with either chromosome 8 or chromosome 18, but not with human chromosome 14 (Tsujimoto *et al.*, 1984b).

We then asked whether the same chromosomal region on chromosome 18 is involved in chromosome translocations in follicular lymphomas. By using two DNA probes approximately 15 kb apart we could detect rearrangements in more than 75% of follicular lymphomas. Therefore we inferred that on band q21 of chromosome 18 there is a gene, for which we propose the name

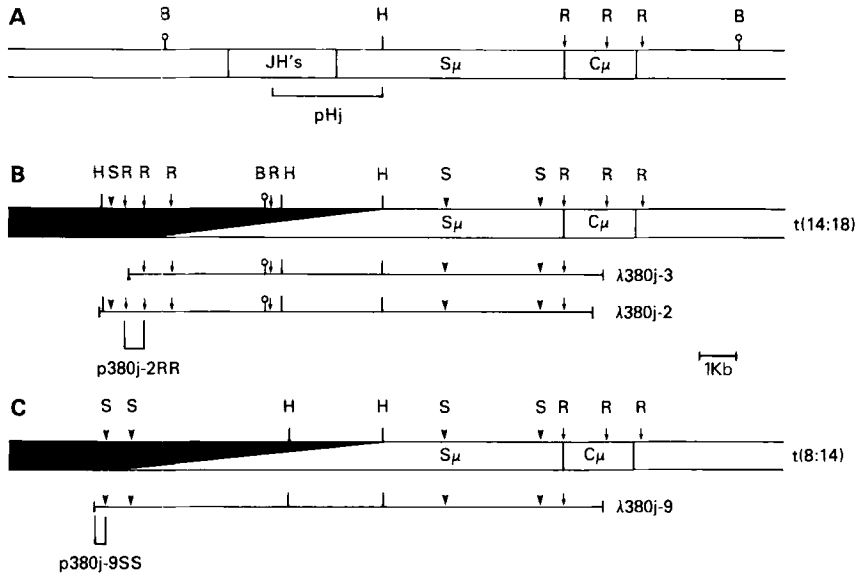

FIG. 14. Restriction maps of the germ line Cμ gene (A) and of the two classes of recombinant clones from the 14q$^+$ chromosomes resulting from the t(14;18) (B) and the t(8;14) (C) translocations. H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI; S, *Sst*I. The black bars represent the chromosome 18-derived sequences in (B) and the chromosome 8-derived sequences in (C). The open bar represents the chromosome 14-derived sequences.

of bcl-2, which is activated because of its proximity to the heavy chain locus (Fig. 15). Since the rearrangements in different neoplastic samples are clustered within a short segment of DNA, it becomes possible to use nucleic acid probes to detect the occurrence of a t(14;18) chromosomal translocation in neoplastic tissues.

Of considerable interest is the fact that we have observed two reciprocal translocations, a t(8;14) and a t(14;18), in the same neoplastic cells of a patient with acute lymphocytic leukemia of the B cell type. We have examined the 380 leukemic cells and found that they are EBV negative, react with monoclonal antibodies specific for B cells, and contain rearranged heavy and light chain genes, but do not express human immunoglobulins (Pegoraro *et al.*, 1984). In the leukemic cells, both μ heavy chain loci are rearranged within the joining (J_H) DNA segment (Fig. 14). One of the J_H segments on one of the 14q$^+$ chromosomes is rearranged with a segment of chromosome 8, 5′ to the c-*myc* oncogene (Tsujimoto *et al.*, 1984b). The c-*myc* oncogene is in its germ line configuration more than 15 kb from the breakpoint and is expressed at elevated levels (ar-Rushdi and Croce, 1985). In addition, the activated c-*myc* transcripts of 380 cells are initiated at the two proper sites

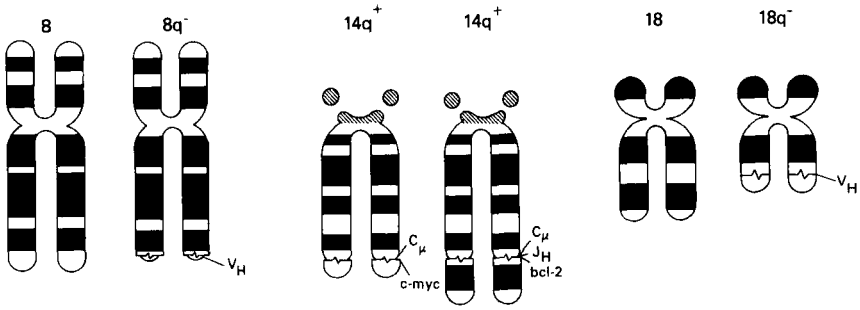

FIG. 15. Diagram of the reciprocal 8;14 and 14;18 translocation carried in 380 cells. Both the bcl-2 locus on band q21 of chromosome 18 and the c-*myc* locus on band q24 of chromosome 8 are translocated in the involved immunoglobulin heavy chain loci on the two chromosomes 14 in 380 cells.

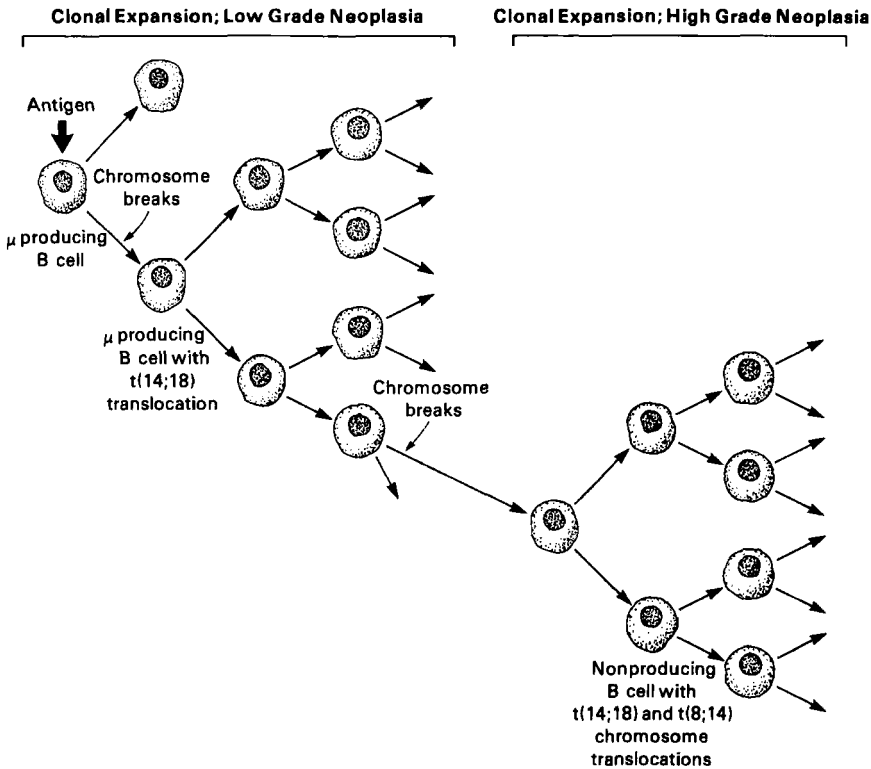

FIG. 16. Model of B cell oncogenesis in B cells carrying two reciprocal translocations involving the two human chromosomes 14 and chromosomes 18 and 8, respectively.

(Nishikura and Croce, 1985). The J_H segment of the other chromosome 14 is rearranged with a segment of chromosome 18 where a putative oncogene, bcl-2, is located (Fig. 15). Based on these findings we propose a model of B cell oncogenesis according to which B cell neoplasms carrying translocations involving the two heavy chain loci are the result of a multiple step process (Fig. 16). The t(14;18) translocation may have occurred in an activated B cell, involving the excluded heavy chain locus on chromosome 14 and the bcl-2 locus on chromosome 18 (Fig. 16). This translocation placed the bcl-2 gene in close proximity to the heavy chain locus and its enhancers leading to the activation and constitutive expression of this putative oncogene (Fig. 15). The consequence of bcl-2 deregulation was the expansion of a malignant clone of B cells carrying the t(14;18) translocation and a relatively low-grade malignancy such as follicular lymphoma (Pegoraro *et al.*, 1984) (Fig. 16). Within the expanded clone of neoplastic B cells, the probability for additional random chromosome rearrangements was increased. One of these additional changes, a translocation between chromosomes 8 and 14, might have occurred in one of the neoplastic B cells (Fig. 16).

This additional translocation then resulted in the activation and constitutive expression of the translocated c-*myc* oncogene, leading to a high grade malignancy such as Burkitt lymphoma or Burkitt-type leukemia (Fig. 16).

In this postulated sequence, the t(14;18) translocation is considered to be an early step in the pathogenesis of the 380 leukemia. A similar role in expanding the pool of proliferating B cells susceptible to the effect of c-*myc* translocation might be ascribed, in other circumstances, to continuous exposure to a strong immunogen such as malaria and/or a virus such as Epstein–Barr virus, that is capable of inducing B cell proliferation. The role of the second translocation [t(8;14)], might be analogous to that of sequential karyotypic alterations in the biological progression of other types of neoplasms, to a more aggressive phase. Thus the study of leukemia and lymphomas carrying two reciprocal chromosomal translocations should allow a better understanding of the sequential steps and multiple genes that may be involved in the pathogenesis of many B cell neoplasms in man.

IX. Conclusions

The studies concerning the involvement of the c-*myc* oncogene in Burkitt lymphoma have indicated that a cellular protooncogene, c-*myc*, can be activated through chromosomal translocations. This activation results from close proximity to genetic elements within the three immunoglobulin loci capable of activating gene transcription in cis in B cells. Thus, the involved c-*myc* oncogene fails to respond to the control mechanisms that are able to regulate

the transcription of the uninvolved c-*myc* oncogene and is expressed constitutively at elevated levels. This derangement in c-*myc* regulation is the common feature of all cases of Burkitt lymphomas and of other B cell malignancies carrying Burkitt-like chromosome translocations (Nishikura *et al.*, 1983; ar-Rushdi *et al.*, 1983; Croce *et al.*, 1983; Erikson *et al.*, 1983a,b). Clearly the c-*myc* product must have a role in the control of B cell proliferation. Its constitutive expression at elevated levels in Burkitt lymphoma cells apparently contributes to their continuous proliferation resulting in a high grade neoplasm.

Consistent reciprocal chromosome translocations involving the heavy chain locus are also observed in other B cell neoplasms of man. For example, the t(11;14) (q13;q32) chromosome translocation is observed in CLL, diffuse B cell lymphoma and multiple myeloma, and the t(14;18) (q32;q21) translocation is observed in follicular lymphomas. Since we have shown that in these cases also the chromosomal breakpoints on chromosome 14 directly involve the heavy chain locus (Erikson *et al.*, 1984; Tsujimoto *et al.*, 1984a,b) it was logical to postulate that specific loci on chromosomes 11 and 18 might be involved in the oncogenic process in malignant B cells carrying these translocations. By "walking" on the $14q^+$ chromosome it has been possible to isolate and characterize the chromosomal regions involved in these rearrangements, providing the tools to isolate and characterize two putative oncogenes, bcl-1 and bcl-2, that might be responsible for many of the B cell neoplasms in man.

Thus specific chromosome rearrangements observed in malignant cells provide us with an opportunity to identify "oncogenes" that are involved in the malignant process which would, otherwise, be undetectable. This approach may result not only in a better understanding of the genes involved in the neoplastic process in B cells, and possibly of the role of these genes in B cell differentiation and/or proliferation, but also provides extremely useful probes to detect specific chromosomal changes in human neoplasms. Thus we can suggest that in the near future the diagnosis of some human B cell malignancies may be carried out by the examination of the DNA of the neoplastic cells using DNA probes flanking the chromosomal breakpoints.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Cancer Institute, CA36521 and CA25875, and grants from the National Foundation–March of Dimes and the American Cancer Society. We thank Kathleen Reinersmann for preparation of this manuscript.

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ADVANCES IN IMMUNOLOGY, VOL. 38

Human Lymphocyte Hybridomas and Monoclonal Antibodies

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I. Introduction

Somatic cell hybridization techniques have changed the scope of immunology. Previously, the preparation of high titer, monospecific antibodies usually required the purification of antigen, the careful immunization of multiple animals, and the repeated absorption of antiserum. The best reagents were jealously guarded by research laboratories; the worst yielded imprecise and irreproducible results. In contrast, one can now generate, with relative ease, lymphocyte hybridomas that secrete monoclonal antibodies against impure and even uncharacterized antigens. The hybridoma antibodies may be prepared in unlimited quantities. They bind to antigen

uniformly and reproducibly, and can mediate a diverse array of biological activities.

Murine hybridoma antibodies have provided crucial information concerning immunoglobulin genetics, and immune regulation. The hybridoma reagents have helped elucidate the structure and function of cell membrane components, lymphokines, enzymes, and many trace proteins. In the diagnostic laboratory, monoclonal antibodies are now used routinely to enumerate lymphocyte subsets, for immunohistological diagnosis, and as reagents for the quantification of serum immunoglobulins and complement proteins. Many other practical applications of murine hybridoma antibodies could be cited.

Clinical immunology research would benefit greatly if methods were available for the reproducible generation of human monoclonal antibodies. On a fundamental level, a comparison of the primary sequences of multiple human monoclonal antibodies against the same antigen would help to delineate the role of immunoglobulin variable region genes, and somatic diversification phenomena, in autoimmune and allergic pathology. With human monoclonals, one could discern if particular clones of autoantibodies also reacted with antigens in the environment, or shared idiotypic structures with antibodies against viruses or bacteria. A clonal analysis of the antigenic specificities of lymphocytes infiltrating the tumor tissues of cancer patients would clarify conflicting data concerning the meaning of immune surveillance for the control of malignant disease.

For *in vivo* therapeutic purposes, human monoclonal antibodies should be much superior to mouse immunoglobulins. The development of an immune response against mouse proteins may neutralize the therapeutic effects of monoclonal antibodies, and trigger potentially dangerous allergic reactions. Human immunoglobulins would be expected to persist in the circulation for longer periods, and would not elicit a vigorous antibody response against constant region determinants.

The potential *in vivo* value of human monoclonal antibodies could include (1) targeting of drugs to foci of cancer cells, to pathogenic microorganisms, and to lymphocytes mediating autoimmune and allergic reaction (Blair and Ghose, 1983; Levy and Miller, 1983), (2) diagnostic imaging to identify malignant cells, damaged tissues, embryologic abnormalities, and infectious agents, (3) passive immunization to prevent hemolytic disease of the newborn, to neutralize toxins and drugs, and to opsonize viruses and bacteria, (4) active immunization with antiidiotypic antibodies to elicit antibodies against pathogens not suitable for conventional vaccines, and (5) modulation of autoimmune and endocrine conditions with antireceptor antibodies. These potential applications, if successful, would have a major impact on human

health. For these reasons, methods for the simple production of human monoclonal antibodies have been pursued vigorously in many laboratories. Unfortunately, the technical hurdles have been considerable, and have hampered progress in the field.

This article will review the accumulating data concerning human monoclonal antibodies and hybridomas. We shall emphasize the special problems that necessarily accompany experiments with people. An attempt will be made to outline possible future directions of human monoclonal antibody research and development.

II. Production of Monoclonal Antibodies by Epstein–Barr Virus-Transformed B Cells

The Epstein–Barr virus (EBV) selectively infects human B lymphocytes that have the CR2 complement receptor on the plasma membrane (Frade *et al.*, 1985). Human and marmoset cell lines have been established that spontaneously release infectious viral particles into the culture medium (Miller and Lipman, 1973). The addition of EBV-containing supernatants to B human lymphocytes induces cell division and transformation (Rosen *et al.*, 1977). The EBV-transformed B lymphocytes express the EB nuclear antigen (EBNA), as detected by anticomplement immunofluorescence (Reedman and Klein, 1973). The infected cells will grow indefinitely in medium lacking exogenous growth factors. Depending on the cell line, EBV transformed human lymphoblasts release IgM, IgG, or IgA into the culture medium (Rosen *et al.*, 1977; Brown and Miller, 1982).

EBV transformation offers a simple alternative to somatic cell hybridization for the immortalization of human antibody-secreting cells. However, several problems have limited the application of the method (Kozbor and Roder, 1983; Cole *et al.*, 1984). Because EBV-infected B lymphocytes are polyclonal, they must be cloned early to preserve specific antibody production. Unfortunately, EBV-transformed normal B lymphoblasts often grow inefficiently at low cell density, even in the presence of feeder layers and exogenous growth factors. Furthermore, EBV infection of unfractionated peripheral blood mononuclear cells triggers a vigorous antiviral response, consisting of α and γ interferon release, and the generation of cytotoxic T cells (Tsoukas *et al.*, 1982; Lotz *et al.*, 1985). To achieve efficacious EBV transformation, it is frequently necessary to remove T lymphocytes prior to infection, or to include a T cell-specific immunosuppressive agent, such as cyclosporin, in the culture medium (Bird *et al.*, 1981).

Despite these obstacles, EBV transformation has been used successfully in many laboratories to produce permanent cell lines secreting human monolconal antibodies. Stable, transformed B cell lines secreting IgM and

TABLE I
ANTIBODY SYNTHESIS BY EBV-TRANSFORMED CELL LINES

Antibody specificity	Class	Concentration (μg/ml)	Reference
Acetylcholine receptor	NA	0.03	Kamo *et al.* (1982)
Chlamydia	IgG	6–13	Rosen *et al.* (1983)
Diphtheria toxin	IgM	NR[a]	Tsuchiya *et al.* (1980)
DNA	IgM	0.1–0.4	Hirano *et al.* (1980)
Hepatitis B	NR	NR	Furuya *et al.* (1982)
4-Hydroxy-3,5-dinitrophenacetic acid	IgM	NR	Steinitz *et al.* (1977)
IgG	IgM	1–2	Steinitz and Tamir (1982)
Influenza virus	IgG	10–20	Crawford *et al.* (1983)
Phosphorylcholine	IgM	NR	Yoshie and Ono (1980)
Pneumococci	IgM	2.8	Steinitz *et al.* (1984)
Rhesus D antigen	IgG	2–50	Koskimies (1980); Boylston *et al.* (1980); Crawford *et al.* (1983)
Sea urchin serum	IgM	8–40	Winger *et al.* (1983)
Streptococci	—	—	Steinitz *et al.* (1979)
Tetanus toxoid	IgM	0.5–1.0	Kozbor and Roder (1981); Zurawski *et al.* (1978, 1980)
Trinitrophenyl	IgM	NR	Kozbor *et al.* (1979)

[a] NR, not reported.

IgG antibodies against viruses, bacteria, and autoantigens, have all been described. Table I summarizes the properties of some EBV-transformed cell lines that have been reported.

As a model system, our laboratory has studied the anti-IgG autoantibody (rheumatoid factor) response induced by EBV transformation (Fong *et al.*, 1982a,b). EBV infection of normal peripheral blood and bone marrow B lymphocytes reproducibly stimulates rheumatoid factor synthesis. Even in patients with rheumatoid arthritis, who have abundant IgG rheumatoid factors, viral transformation of peripheral blood B cells induces predominantly autoantibodies of the IgM class. Moreover, the rheumatoid factors in the supernatants of EBV-transformed B cell lines and in the serum of the lymphocyte donors express different idiotypes (Pasquali *et al.*, 1981). In contrast, stimulation of peripheral blood lymphocytes from rheumatoid arthritis patients with pokeweed mitogen yields anti-IgG autoantibodies with idiotypic antigens similar to serum rheumatoid factors. These experiments suggest that EBV may preferentially stimulate a minor subset of B cells that often is not expressed *in vivo*. Indeed, it is clear that only a small proportion of B lymphocytes with CR2 receptors grow out after superinfection with

EBV. Cells with surface IgM have a high EBV receptor density and are infected preferentially (Brown and Miller, 1982). The precursor frequency of B cells activated by EBV has been reported to range from 1/290–1/3700 for IgM, and 1–920–1/3250 for IgG (Martinez-Maza and Britton, 1983).

III. Cell Lines for B Lymphocyte Hybridization

Antibody-producing hybridomas may be created by the somatic fusion of normal lymphocyte or plasma cells with established lymphoblastoid or plasma cell lines. In the resulting heterokaryons, the normal lymphocytes provide the genetic information for the synthesis of specific antibody. The malignant cells confer immortality. The success of the technique requires the stable maintenance of both qualities in the somatic cell hybrids.

The requirements for the production of hybridomas secreting human monoclonal antibodies are basically the same as in murine systems (Kohler *et al.*, 1976; Kohler and Milstein, 1975). The fusion partners must have similar phenotypes. Otherwise, the differentiated functions of both parental cell types will not be maintained. For instance, the fusion of lymphocytes with fibroblasts or anaplastic cells permits only transient immunoglobulin mRNA synthesis. Indeed, somatic cell hybridization is still used extensively to study tissue specific regulation of gene expression. Both normal and malignant lymphoblasts and plasma cells will support immunoglobulin synthesis and secretion, to varying degrees. Antibody-secreting human hybridomas could therefore derive from fusion of immune lymphocytes with lymphoblastoid or plasma cell lines.

The hybridomas must have relatively stable karyotypes. Otherwise, a selection pressure is needed to prevent the loss of the genes encoding immunoglobulin light and heavy chains. Human–mouse somatic cell hybrids preferentially expel human chromosomes (Ruddle, 1974). The structural genes for human heavy chains are on chromosome 14; the genes for κ and λ chains are on chromosomes 2 and 22, respectively (Croce *et al.*, 1979; Erickson *et al.*, 1981; Malcom *et al.*, 1982). Chromosomes 14 and 22 may persist in human–mouse lymphoid hybridomas longer than other chromosomes (Croce *et al.*, 1980b). However, the presence of chromosome 14 does not ensure the continued synthesis of human heavy chains by the hybridoma.

The parental tumor cell line must have a high frequency of hybrid formation (preferably at least 1 in 10^5 cells). It must clone at high efficiency, and should grow quickly. The cell line must also carry one or two biochemical markers that can be exploited to eliminate surviving parental cells. In the system developed originally by Littlefield (1964) the parental cell line is treated with increasing concentrations of 8-azaguanine or 6-thioguanine. Cells containing the enzyme hypoxanthine-guanine phosphoribosyltrans-

ferase (HGPRT) incorporate the purine antimetabolites into nucleic acid. Only HGPRT-deficient cells survive the drug treatment. The HGPRT gene is on the X chromosome in mammals, and therefore yields mutants with high frequency. The number of mutants may be increased by prior incubation of the cells with alkylating agents, or by exposing them to ionizing radiation.

In normal tissue culture media, the HGPRT enzyme is not necessary for growth. However, in medium supplemented with an inhibitor of *de novo* purine synthesis, such as aminopterin, amethopterin, or azaserine, only HGPRT-positive cells can utilize exogenous hypoxanthine to satisfy purine requirements. HGPRT-deficient cell lines, after fusion with normal lymphocytes or plasma cells, will grow in selective medium containing hypoxanthine–aminopterin–thymidine (HAT) or hypoxanthine–azaserine (HA), only if they have acquired the genetic information for the synthesis of a functional HGPRT protein from the normal lymphocyte parent. HAT medium includes thymidine, because antifolates also block thymidylate synthetase. It is not necessary to add thymidine to azaserine-supplemented HA medium (Lakow *et al.*, 1983; Foung *et al.*, 1982).

Normal murine B lymphocytes or plasma cells will not proliferate indefinitely in tissue culture, at least in the absence of exogenous growth factors. Therefore, no selection pressure is usually necessary to eliminate residual normal lymphocytes after hybridization. However, human B lymphocytes from normal donors may harbor the genome of the Epstein–Barr virus. After depletion of regulatory T cells, monocytes, and natural killer cells, B cells containing EBV genetic material may transform spontaneously and develop into permanent immunoglobulin secreting cell lines (Bird *et al.*, 1981). The EBV-transformed cells will grow in HAT or HA medium. Thus, it is necessary to prove rigorously that human monoclonal antibody-secreting cell lines are hybridomas, rather than EBV-transformed diploid lymphoblasts.

Methods have been devised to eliminate EBV-transformed B lymphoblasts, and other parental cells that survive the hybridization procedure. Resistance to micromolar concentrations of ouabain, a potent inhibitor of Na^+,K^+-ATPase, is a natural property of most murine cell lines, but not human cells. Ouabain resistance behaves as a dominant trait. HAT sensitivity is recessive (Kozbor *et al.*, 1982b). Secondary mutants resistant to ouabain may be selected with relative ease from HGPRT-deficient human lymphoblastoid and plasma cell lines (Lakow *et al.*, 1983; Kozbor and Roder, 1984). HAT-sensitive, ouabain-resistant cells are universal hybridization partners. In HAT–ouabain medium, only cells that have acquired a functional HGPRT gene from the normal parent, and a mutant ATPase from the malignant parent, can proliferate.

It is also possible to introduce a dominant drug marker into human or murine lymphoblastoid cell lines by DNA transfection, or by infection with

TABLE II

CELL LINES USED FOR CONSTRUCTION OF HYBRIDOMAS SECRETING HUMAN ANTIBODIES

Species	Cell line	Derivation	Ig secretion	Reference
Mouse	P3-X63-AG8	P3-X63	IgGK	Levy and Dilly (1978)
	NS-1	P3-X63	Light chain	Schlom, Wunderich, and Teramoto (1980)
	P3-X63-AG8.653	P3-X63	Nonsecretor	Gigliotti and Insel (1982)
	SP-1	P3-X63	Nonsecretor	Butler, Lane, and Fauci (1983)
	NSO/U	NS1/1.Ag4.1	Nonsecretor	Galfre and Milstein (1982)
	SP-2/0 Ag14	P3-X63	Nonsecretor	Astaldi *et al.* (1982)
	DR21-40B5	P3-X63	Nonsecretor	Cieplinski *et al.* (1983)
Rat	YB2/3.0 Ag20	Rat/Y3 hybrid	Nonsecretor	Galfre and Milstein (1982)
Human	SK-007	U-266	IgEλ	Olsson and Kaplan (1980)
	FU-266	U-266	IgEλ	Teng *et al.* (1983)
	U-266-Az^R	U-266	IgEλ	Abrams *et al.* (1983)
	8226-AzR	RPMI-8226	λ	Abrams *et al.* (1983)
	8226AR/NIP4-1	RPMI-8226	None	Pickering and Gelder (1982)
	LICR-LON-HMy2	ARH-77	IgG_1K	Edwards *et al.* (1982)
	GM-1500-6TG-AL-1	GM-1500	IgG_2K	Croce *et al.* (1980)
	GM-1500-6TG-AL-2	GM-1500	IgG_2K	Croce *et al.* (1980)
	KR-4	GM-1500	IgG_2K	Kozbor, Lagarde and Roder (1982)
	GM-4672	GM-1500	IgG_2K	Shoenfeld *et al.* (1982)
	GK-5	GM-1500	IgG_2K	Dwyer *et al.* (1983)
	GM-0467	PGLC 33H	IgMλ	Chiorrazi, Wasserman, and Kunkel (1982)
	UC-729-6	WIL-2	IgM_K	Glassy *et al.* (1983)
	UC-729-HF2	WIL-2	IgM_K	Heitzman and Cohn (1983)
	LTR-228	WIL-2	IgM_K	Larrick *et al.* (1983)
	H351.1	WIL-2	IgM_K	Chiorrazi, Wasserman, and Kunkel (1982)
	MC/ZNS-1.2.3	MCCAR	None	Ritts *et al.* (1983)
	RH-L4-AG.8	RH-L4	None	Brodin, Olsson, and Sjogren (1983)
	LSM1.1	H.S. Sultan	None	Posner, Schlossman, and Lazarus (1983)
	LSM1.2	H.S. Sultan	None	Posner, Schlossman, and Lazarus (1983)
	HFB-1	HFB-1	None	Hunter *et al.* (1982)
Mouse/human	SPAZ-4	SP-2/human PBL	None	Ostberg and Pursch (1983)
	D-33	FU-266/X63-Ag8.653	None	Teng *et al.* (1983)
Human/human	HM 2.0	LSM 1.2/plasma cell leukemia	None	Posner, Schlossman, and Lazarus (1983)
	LSM 2.7	LSM 1.1/human PBL	None	Schwaber *et al.* (1984)

an amphotropic retrovirus. Plasma cell lines have been rendered resistant to the cytotoxic antibiotic G418 by introduction of a plasmid vector (pSV2-neo^R) containing a bacterial neomycin resistance gene linked to a Simian virus 40 (SV40) promoter (Foung *et al.*, 1984; Teng *et al.*, 1983; Riera *et al.*, 1984).

Ideally, the lymphoblast or plasma cell line used for fusion should not synthesize immunoglobulin. Otherwise, the joining of two antibody-secreting cells will yield hybridomas producing monoclonal antibodies composed of molecules containing light and heavy chains from both parental cells. IgM molecules contain 10 light and 10 heavy polypeptide chains. In principle, IgM hybridomas derived from two immunoglobulin-secreting parental cells could synthesize a very large number of different immunoglobulin variants.

In sum, a preferred cell line for fusion with normal human B lymphocytes or plasma cells should (1) support antibody production, and not secrete immunoglobulin, (2) have a high frequency of hybridoma formation, (3) clone efficiently and grow quickly, (4) possess easily selectable dominant and recessive biochemical markers, and (5) yield hybridomas with stable karyotypes and phenotypes. To date, no cell line has been isolated with all these characteristics. Table II lists the mouse and human lymphoblastoid cell lines, and plasmacytoma cell lines, that have been used to generate human antibody-secreting hybridomas.

IV. Preparation of Donor Lymphocytes for Fusion

The keys to successful human monoclonal antibody production by somatic cell hybridization are the quantity, purity, and state of activation of the donor lymphocytes. The donor cells must be metabolically active, and highly enriched in cells producing the antibody of interest. Usually, B lymphocytes with both these characteristics are exceedingly difficult to obtain in quantity. Safety and ethical considerations have prevented the routine immunization of humans solely for scientific purposes. The sources of donor lymphocytes that have yielded successful human monoclonal antibody-producing hybridomas include (1) homogeneous populations of malignant B lymphocytes from patients with chronic lymphatic leukemia, lymphoma, or Waldenstrom's macroglobulinemia, (2) normal splenic lymphocytes from patients who have been vaccinated prior to exploratory laparotomy and splenectomy, (3) draining lymph nodes adjacent to surgically removed tumors, and (4) peripheral blood lymphocytes from normal donors and patients with various diseases. The malignant B lymphocytes, and the lymphocytes that have been primed *in vivo*, will fuse efficiently and yield hybridomas without further *in vitro* stimulation.

The peripheral blood provides readily accessible donor lymphocytes.

However, is not a major site of immunoglobulin-secreting cells in humans. Only 5–10% of peripheral blood lymphocytes have surface Ig. Most of these B cells are in a resting, or G_0 state. Resting lymphocytes are not fully competent metabolically. The few circulating plasma cells are probably terminally differentiated, with a minimal growth potential. The fusion of G_0 or terminally differentiated cells with dividing lymphoblasts or plasmacytoma cells will yield cytoplasmic hybridomas. However, the nuclei frequently will not fuse, and the multinucleate cells will not divide. By contrast, if the normal donor lymphocytes are in a proliferative state, nuclear and cytoplasmic fusion occurs more readily, and the hybridomas replicate.

Human peripheral B blood lymphocytes will proliferate after exposure to antigens, in the presence of accessory macrophages and T lymphocytes. However, methods for the primary *in vitro* immunization of human peripheral blood lymphocytes with soluble antigens are not well defined (Astaldi *et al.*, 1982; Cavagnaro and Osband, 1983; Foon *et al.*, 1983; Morimoto *et al.*, 1981). Several techniques have been described, but none has achieved widespread usage among immunologists.

To activate human peripheral blood lymphocytes in a reproducible manner, one must either immunize patients *in vivo*, or nonspecifically stimulate peripheral blood lymphocytes *in vitro* with a B cell mitogen. The content of antigen-specific B lymphocytes in the circulation depends critically upon the time postimmunization that blood samples are removed. For example, several distinct subsets of B cells appear sequentially following a booster injection of normal volunteers with tetanus toxoid (Butler *et al.*, 1983; Geha, 1981; Saxon *et al.*, 1982; Stevens *et al.*, 1979). Between 5 and 10 days after immunization, one obtains large B lymphocytes and plasma cells that spontaneously secrete IgG antitetanus toxoid antibodies *in vitro*, without a requirement for either T cells or pokeweed mitogen. These cells are devoid of surface immunoglobulin, complement receptors, and Fc receptors, but may express Ia antigens. About 2 weeks after immunization, this lymphocyte population disappears and a second group of smaller B cells emerges. The latter lymphocytes are capable of producing IgG antitetanus toxoid antibody, but require pokeweed mitogen, or antigen and T cell help, for maximum immunoglobulin synthesis. The inducible cells remain in the circulation for variable lengths of time. Gradually, they become unresponsive to antigen, but retain the capacity to respond to nonspecific mitogens.

Spontaneous IgM antitetanus toxoid antibody synthesis is minimal after secondary immunization. The frequency of pokeweed mitogen-inducible IgM antitetanus toxoid B cells also remains relatively unchanged. Lymphocytes capable of producing IgE antitetanus toxoid antibody appear between days 19 and 25 days postimmunization. The synthesis of specific IgE antibody requires both pokeweed mitogen and T cell help. It is only by appre-

ciation of such complex transitions in the B cell repertoire that one can effectively utilize peripheral blood lymphocytes from immunized volunteers as a source of antigen-specific B cells for fusion.

Resting peripheral blood lymphocytes will divide and differentiate *in vitro* after activation with pokeweed mitogen, killed *Staphylococcus aureus* Cowan strain 1 (SAC), Nocardia water soluble antigen, the Epstein–Barr virus (EBV), and antiimmunoglobulin antibodies (Moller, 1979). Prior stimulation of peripheral blood mononuclear cells with pokeweed mitogen, SAC, or EBV has been reported to increase the frequency of B lymphocyte fusion by 10- to 100-fold (Heitzmann and Cohn, 1983; Kozbor and Roder, 1984; Freimark, 1985; Freimark and Ozer, 1985). The enhancement may relate in part to mitogen-induced increases in the negative charge of the plasma membrane (Toister and Layter, 1971). Calcium ions and other divalent cations provide an ionic bridge between the activated lymphocytes.

Limiting dilution techniques indicate that only minute numbers of human peripheral blood B lymphocytes are capable of responding to any given antigen. For instance, in many rheumatoid arthritis patients, the frequency of IgM rheumatoid factor producing B cells is only 1 in 10^4 or 1 in 10^5 peripheral blood lymphocytes (Fong *et al.*, 1982b). Similarly, in recently vaccinated volunteers, the frequency of cells producing antitetanus toxoid antibody is only 1 in 10^4 peripheral blood lymphocytes (Stevens *et al.*, 1979; Saxon *et al.*, 1982). The fusion frequency of currently available human–human and human–mouse hybridoma systems is no more than 1 in 10^5 cells (Foon *et al.*, 1983; Abrahms *et al.*, 1983; Heitzmann and Cohn, 1983). Thus, 10^9 to 10^{10} peripheral blood lymphocytes probably are needed to generate a single antigen-specific hybridoma. Leukophoresis provides the only practical method for obtaining peripheral blood B cells in these quantities.

Several investigators have attempted to enrich for antigen-specific B lymphocytes prior to cell fusion. Steinitz *et al.* (1977) described a rosetting technique for the purification of peripheral blood B lymphocytes reactive with the synthetic hapten NNP (4-hydroxy-3,5-dinitrophenacetic acid). Peripheral blood lymphocytes were incubated with NNP coupled to autologous erythrocytes or type O negative red blood cells. Subsequent centrifugation through Ficoll–Hypaque gradients separated the hapten-binding B lymphocytes from the remainder of the peripheral blood mononuclear cell fraction. Infection with EBV yielded a transformed cell line that secreted anti-NNP antibody. A similar rosetting approach facilitated the partial isolation of B cells reactive with rhesus antigen D, streptococcal carbohydrate A, IgG Fc fragments, and tetanus toxoid (Steinitz *et al.*, 1979a,b; Steinitz and Tamir, 1982).

Kozbor and Roder (1981) applied a negative preselection technique to concentrate tetanus toxoid specific B cells from the peripheral blood of

immunized volunteers. A crude mononuclear cell fraction was exposed to soluble tetanus toxoid, to induce the capping and internalization of specific antibody receptors on the plasma membrane. Then the residual surface immunoglobulin positive cells were depleted by rosetting with antiimmunoglobulin coated red blood cells, followed by isopycnic centrifugation. The activated, surface immunoglobulin-negative cells were transformed with EBV. Eventually they were fused with human or murine plasmacytoma cells. Consistent with their proliferative state, the EBV-activated B lymphoblasts had a higher fusion frequency than normal resting peripheral blood B cells.

Another approach for the purification of antigen-specific B lymphocytes utilizes cell "panning" (Fong *et al.*, 1981). With this method, antigens (or antibodies) are adsorbed onto plastic petri dishes. Peripheral blood lymphocytes are exposed to the coated plates. The nonadherent cells are gently removed, leaving behind an enriched fraction of antigen-binding lymphocytes. Winger *et al.* (1983) have used this technique successfully to enrich for B lymphocytes reactive with sea urchin sperm antigens. After EBV transformation, they obtained lymphoblastoid cell lines secreting specific anti-sea urchin sperm antibody.

A novel technique to increase specific hybrid formation was recently described by Lo and colleagues (1984) in a murine model. The investigators exposed B lymphocytes to biotin-coupled antigen. After removal of excess conjugates, the B cells were incubated at 4°C with cultured myeloma cells, to which avidin had been linked covalently. Biotin binds avidin with extremely high affinity. The antigen-binding B cells preferentially adhered to the myeloma cells via antigen–biotin–avidin bridges. Fusions performed after this initial focusing step yielded a large number of hybrids secreting specific antibody. The same method should be applicable to human hybridization protocols.

The fluorescence-activated cell sorter permits one to select rare antibody-secreting murine hybridomas from mixed cell populations. The technique requires fluorescent antigen and state-of-the-art sterile flow cytometry. It is time consuming and extremely expensive. Nonetheless, the power of the method warrants its use for human hybridization experiments.

Preincubation of human peripheral blood lymphocytes with antigen has been reported to increase the frequency of antigen-specific hybridomas by 2- to 5-fold at most. This unimpressive result is not surprising, considering the low frequency of precursor B cells for any antigen in the peripheral blood, and the current lack of purified human B cell growth and differentiation factors. The cloning and expression of the genes for human B cell growth and differentiation factors may be achieved in the near future (Okada *et al.*, 1983). The availability of the purified lymphokines, along with improved

methods for isolating antigen-binding B cells, may render possible the primary *in vitro* immunization of human peripheral blood lymphocytes.

The frequency of human peripheral blood T lymphocytes that recognize allogeneic histocompatibility antigens is extraordinarily high. After fusion of unfractionated human peripheral blood mononuclear cells with an HLA nonidentical lymphoblastoid cell line, the surviving T cells may react with and destroy the heterokaryons. This theoretical problem has not hampered human–human hybridization experiments. However, it may be avoided by removing the T lymphocytes prior to fusion, or by treating the cultures with cyclosporin. Alternatively, a ouabain (or G418)-resistant, HAT-sensitive lymphoblastoid cell line may be used for fusion. In HAT–ouabain medium, only true hybridomas can proliferate.

The lymph nodes, spleen, and bone marrow of humans represent significant sources of IgG antibody-secreting cells. In several instances, monoclonal antibodies have been generated by the fusion of splenic or lymph node B cells from volunteers injected with antigen prior to medically necessary surgery (Olsson and Kaplan, 1980). Surprisingly, bone marrow cells have not been used for human monoclonal antibody production. Large numbers of bone marrow lymphocytes may be obtained safely by needle aspiration, or during routine hip replacement surgery. This ready source of *in vivo* activated B lymphocytes deserves further attention.

To reiterate, the difficult procurement of antigen-specific, activated B lymphocytes has hindered severely human monoclonal antibody research and development. The frequency of antigen-specific B cells in human peripheral blood is low. Methods for primary *in vitro* immunization of human peripheral blood lymphocytes are inadequate. Resting B lymphocytes, and terminally differentiated plasma cells, fuse inefficiently. Nonspecific suppressor T lymphocytes, and T cells directed against allogeneic histocompatibility antigens, may impede the growth of hybridomas. Attempts have been made to overcome each of these problems. *In vitro* stimulation with polyclonal B cell activators, the preselection of antigen-binding B cells, and the removal or inactivation of T lymphocytes, have all been of modest benefit. Hybridomas have been generated reproducibly from the lymph nodes and spleens of volunteers immunized deliberately *in vivo*, as well as from the malignant lymphoblasts of patients with antibody-secreting B cell malignancies.

V. Cell Fusion

Currently, polyethylene glycol (PEG)-induced cell fusion is the most common method used to generate human hybridomas (Brahe and Serra, 1981). When cells are suspended for brief periods in concentrated solutions of

PEG, membrane phospholipids are dehydrated (Arnold *et al.*, 1983). The reduction in free water content leads to closer apposition of cell membranes. The degree of cell fusion is influenced by the concentration of the PEG solution and the molecular weight of the polymer. Human hybridomas have been generated with PEG solutions ranging from 35 to 50% (w/v) with molecular weights ranging from 1000 to 6000. Most often a 50% (w/v) solution of molecular weight 4000 PEG has been employed.

Despite many attempts to increase the fusion yield, only about 1 in 10^5 human lymphocytes form hybrids. Recently, a new method of cell joining, called electrofusion, was developed (Zimmerman, 1982; Zimmerman and Vienken, 1982). The technique requires the mixing of myeloma cells and lymphocytes between two electrodes in a sterile chamber. After application of an alternating voltage of about 100 V/cm with a frequency of 1 MHz, polarization of the cells causes a "pearl chain" to form between the electrodes. Once the correct cells are in close proximity to one another, a stronger field pulse of about 4 kV/cm induces cell fusion. In principle, this technique can generate very high fusion efficiencies with very few input cells required (Claude and Justin, 1983). More experiments will be needed to determine its full potential.

Most protocols for the generation of human hybridomas use feeder cells to promote the outgrowth of unstable heterokaryons after the primary fusion. Additionally, feeder cells enhance the cloning efficiency of selected hybrids. Human monocytes, human peripheral blood lymphocytes, mouse thymocytes, mouse peritoneal macrophages, and established human cell lines have all been utilized. The benefits of the different cell types have not been compared systematically.

VI. Human–Murine Hybridomas

A. Rescuing Immunoglobulin Secretory Capacity by Human–Mouse Somatic Cell Hybridization

In 1973, Schwaber and Cohen first generated human–murine heterohybridomas that secreted human immunoglobulin. TEPC-15 mouse myeloma cells were fused with human peripheral blood lymphocytes. Fluorescent antibody methods indicated that the resulting heterokaryons synthesized both mouse and human immunoglobulins. The culture supernatants contained mixed immunoglobulin molecules composed of human and murine heavy and light chains. Subsequently, these and other investigators used human–mouse lymphocyte hybridization to probe the metabolic defects in B cells of children with hypogammaglobulinemia (Schwaber and Rosen, 1978;

Schwaber, 1975; Denis *et al.*, 1983). In several instances, hybridomas were isolated that secreted abundant human immunoglobulin. The results clearly demonstrated that fusion with plasmacytoma cell lines could restore immunoglobulin secretion by the B cells from immunodeficient patients.

In a later study, Levy and Dilley (1979) fused B leukemic cells from four patients with the P3-X63-Ag8 murine myeloma cell line used by Kohler and Milstein (1975). Among 200 different hybridomas cultures that were analyzed, 57% secreted human immunoglobulin, or mixed molecules composed of human and mouse heavy and light chains. Some of the hybridomas produced antibody during several months of continuous culture. Importantly, the donor B leukemia cells expressed immunoglobulin on the plasma membrane, but failed to secrete significant quantities of antibody. Apparently, the phenotype of the murine partner was dominant in the hybrids and provided the metabolic machinery for human immunoglobulin synthesis.

The human immunoglobulin in the heterohybridoma culture supernatants was purified by antibody affinity chromatography. The isolated protein elicited specific mouse antiidiotypic antibodies that reacted with the malignant B cells. Such custom-designed antiidiotypes have been administered to patients with chronic lymphatic leukemia, in an effort to induce antibody-mediated tumor regression (Levy and Miller, 1983).

B. Heterohybridoma Antibodies against Bacteria, Viruses, and Toxins

Human monoclonal antibodies against bacteria, viruses, and toxins would have considerable clinical utility for passive immunization. Nowinski and coworkers (1980) exposed human splenic lymphocytes *in vitro* to influenza virus. The cells were fused with the NS-1 variant murine myeloma line. Heterohybridomas were isolated in HAT medium, and the culture supernatants were screened for antiinfluenza antibodies. After repeated subcloning, one cell line was isolated that produced an IgM κ monoclonal antibody reactive with three out of five influenza strains. Surprisingly, the monoclonal immunoglobulin also bound the Forssman antigen. Such cross-reactivity and relative lack of specificity has been a recurrent problem with human IgM monoclonal antibodies generated in the absence of deliberate *in vivo* immunization.

Gigliotti and Insel (1982) vaccinated normal volunteers with diphtheria and tetanus toxoids. One week later, peripheral blood lymphocytes were removed and were fused with the P3-X63-Ag8.653 nonsecreting murine myeloma variant (Kearney *et al.*, 1979). Many human immunoglobulin secreting heterohybrids initially grew out. Once again, repeated subcloning was required to maintain antibody production. Eventually, four hybridomas were isolated that released IgG antitetanus toxoid antibody during several months continuous culture.

The same group immunized normal adults with a vaccine containing the purified capsular polysaccharide of *Haemophilus influenzae* type b (Gigliotti *et al.*, 1984; Insel, 1984). Seven days later a B cell-enriched fraction was prepared from the peripheral blood mononuclear cells, and was fused with P3-X63-Ag8.653. Specific antibody-secreting heterohybridomas were subcloned by limiting dilution over mouse peritoneal macrophage feeder cells. Human immunoglobulin levels in the culture supernatants were reported to reach 5 μg/ml. Twelve of thirteen rats pretreated with 70 μg of the human monoclonal antibody survived intraperitoneal injection with 10^7 *Haemophilius influenzae* organisms.

Kozbor and colleagues (1981, 1982a) combined Epstein–Barr virus transformation with human–mouse somatic cell hybridization. Peripheral blood lymphocytes from a tetanus toxoid immunized individual were infected with the B95-8 strain of Epstein–Barr virus. A cell line secreting an IgM κ antitetanus toxoid antibody emerged. The uncloned virus transformed cells were fused with the P3-X63-Ag8.653 murine myeloma. Subsequent culture in ouabain–HAT medium eliminated the unfused human parental cells as well as the HGPRT-deficient murine myeloma cells. The heterohybridoma cell lines were reported to produce the same IgM κ monoclonal antibody as the parental EBV line, and remained stable during 6 months passage *in vitro*.

Butler *et al.* (1983) attempted to define optimal conditions for mouse–human hybridization utilizing the peripheral blood cells of immunized subjects. Normal volunteers received booster injections with tetanus toxoid. Blood lymphocytes were isolated at various intervals thereafter. The mononuclear cells were fused with the HAT-sensitive SP-1 murine hybridoma. Lymphocytes obtained 5–7 days after immunization yielded the highest number of heterohybridomas secreting specific antitetanus toxoid antibody (8.5% positive wells at 5–7 days compared to <1% at either 3 or 14 days). Lymphocytes cultured *in vitro* for 3 days with tetanus toxoid prior to fusion yielded approximately twice as many hybridomas. The optimal fusion ratio was two peripheral blood lymphocytes to one myeloma cell. Seeding 300,000 cells in each 200 μl microculture well was optimal for hybridoma growth. The authors concluded that stable mouse–human heterohybridomas could be isolated (1) by adopting an early aggressive approach to cloning, and (2) by paying careful attention to the complex kinetics of the circulating B cell population in the peripheral blood after immunization with a soluble antigen. In general, other investigations have supported these conclusions.

C. Antibodies against Tumors Produced by Human–Mouse Heterohybridomas

The availability of hybridoma technology has prompted tumor immunologists to reanalyze the human immune response against autologous ma-

lignant cells. Schlom and co-workers (1980) isolated lymphocytes from the draining axillary lymph nodes of breast cancer patients. After fusion with NS-1 murine myeloma cells, heterohybridoma supernatants were screened by immunofluorescence for binding to breast tumor tissue. Several supernatants gave initially positive results. However, all ceased to secrete human antibody with continued propagation *in vitro*.

In an extensive study, Cote *et al.* (1983) obtained lymphocytes from the lymph nodes, peripheral blood, spleen, and biopsy specimens of cancer patients. The lymphocytes were hybridized with the NS-1 mouse myeloma line and also with two human lymphoblastoid cell lines. About half of the human–mouse heterohybridomas secreted immunoglobulin for 6 to 7 months in tissue culture. One heterohybridoma derived from a fusion between NS-1 and the axillary lymph node lymphocytes of a patient with breast cancer. The cell line produced an IgG antibody reactive with a plasma membrane antigen on several cancer cell lines and on mononuclear cells from peripheral blood. The concentration of specific antibody in the culture supernatant reached 5 μg/ml.

Sikora *et al.* (1982, 1983) carefully removed the lymphocytes from a brain tumor specimen, and fused the cells with the NS-1 murine myeloma. Some of the resulting heterohybridomas produced IgG that reacted with autologous tumor, as detected by immunofluorescence. However, none was stable during continued culture. In a similar study, the same authors hybridized lymph nodes from a lung cancer patient with mouse NS-1 myeloma cells, and with rat Y3-Ag.123 myeloma cells (Sikora and Phillips, 1981; Sikora and Wright, 1981). Although some of the heterohybrids again displayed antitumor reactivity, as detected by immunofluorescence, their instability precluded further detailed analysis.

Glassy *et al.* (1983a,b) isolated lymphocytes from the draining lymph nodes of patients with prostate carcinoma, and fused the cells with NS-1. One heterohybridoma (MGH-7) was reported to secrete a monoclonal antibody reactive with two prostate carcinoma cell lines and with frozen sections of prostate carcinomas.

It is not clear whether or not the various monoclonal antibodies secreted by the human–mouse heterohybridoma were actually tumor specific. Apparently, few or none of the hybridomas have been stable enough, or available in sufficient quantity, to permit a standard analysis of antigenic specificity. Neither immunoprecipitation nor western blotting studies have been reported. Moreover, the antibodies secreted by the heterohybridomas have not been compared with the patients' circulating immunoglobulins.

D. Other Antigens

Lane and colleagues (1982) immunized normal volunteers with keyhole limpet hemocyanin (KLH). Ten days later, the peripheral blood mono-

nuclear cells were fused with the SP-1 murine hybridoma. Among 240 culture wells, each seeded with one million cells, eight secreted IgM or IgG anti-KLH antibody. One stable anti-KLH subclone released up to 30 μg/ml IgM.

Galfre and Milstein (1982) fused human spleen cells with the nonsecretor NSO/U mouse myeloma, and human peripheral blood mononuclear cells with the nonsecretor rat YB2/3.0AG20 myeloma. After a month in culture, one human–mouse heterohybrid (NG9) continued to secrete κ light chains, and remained stable thereafter. Sufficient quantities of light chain were obtained for N-terminal sequence analysis.

E. Advantages of Mouse–Human Hybridization

Antibody-secreting human–mouse heterohybridomas have been produced in several laboratories, utilizing diverse donor lymphocyte sources and several different nonsecreting murine plasma cell lines. In general, successful heterohybridomas originated from the peripheral blood or splenic lymphocytes of individuals immunized *in vivo* prior to cell fusion. Typically, the heterohybridomas ceased to secrete specific antibodies after 1 to 2 months in tissue culture. Factors contributing to the decline in immunoglobulin production were the preferential loss of human chromosomes, and a poorly understood specific suppression of human immunoglobulin secretion. With repeated subcloning, it nevertheless was possible to stabilize human immunoglobulin production in several heterohybridoma cell lines. Even in the absence of permanent antibody secretion, human–mouse heterohybridomas have permitted genetic complementation studies with nonsecreting B lymphocytes from patients with agammaglobulinemia, chronic lymphocytic leukemia, and lymphoma.

Excluding the products of triple fusions, human–mouse B cell heterokaryons should contain one, or at most two, rearranged human heavy and light chain genes. Hence, they provide convenient starting material for the isolation of antibody gene segments. Recently, Bentley (1984) cloned and sequenced a human κ chain cDNA from the NG9 human–mouse heterohybridoma. With improved methodology, perhaps sufficient human immunoglobulin mRNA for cloning could be obtained from even unstable heterohybridomas cell lines. Eventually, the isolated cDNA for the heavy and light chains could be reintroduced into murine myeloma cells with appropriate vectors.

The ideal murine plasmacytoma cell line for human–mouse fusion would have drug markers to preserve human chromosomes 14 (heavy chain), two (κ chain) and 22 (λ chain). Two potentially selectable enzymes, that are encoded by genes on human chromosome 14, are purine nucleoside phosphorylase and nicotinic acid phosphoribosyltransferase (George and Francke, 1976; Elliott, 1981). Purine nucleoside phosphorylase-deficient murine

cell lines have been selected by two groups (Ullman *et al.*, 1979; Hoffie *et al.*, 1983). In medium containing low methionine plus methylthioinosine, low glucose plus inosine, or azaserine plus inosine, cell growth depends upon a functional nucleoside phosphorylase enzyme (Hoffie *et al.*, 1983; Carson *et al.*, 1983). Although not yet described in a mammalian system, nicotinic acid phosphoribosyltransferase-deficient cells could be selected by mutagenesis and exposure to a toxic nicotinic acid analog. Lymphocytes do not produce significant NAD from tryptophan. In tissue culture medium lacking nicotinamide, but supplemented with nicotinic acid, the synthesis of NAD depends entirely upon nicotinic acid phosphororibosyltransferase. Selectable markers for chromsomes 2 and 22 have not yet been devised. Conceivably, transfection with anti-sense RNA coupled to an inducible promoter could be used to construct murine myeloma cell lines with conditionally lethal deficiencies in enzymes encoded by genes on human chromosomes 2 and 22. These approaches to human–mouse hybridization remain topics for future research.

VII. Human–Human Hybridomas

A. Cell Lines for Fusion

Because human–mouse heterohybridomas are unstable, investigators have turned to human lymphoblastoid and myeloma cell lines as alternatives for cell fusion. Table II summarizes the characteristics of several cell lines that have yielded hybrids producing monoclonal antibodies. None of the cell lines is entirely satisfactory. Problems with the human cells have included (1) poor hybridization and cloning efficiency, (2) production of only very small amounts of monoclonal antibody, (3) presence of Epstein–Barr viral genes capable of transforming unfused B cells, (4) inability to isolate non-secreting human myeloma cell variants comparable to the murine plasmacytomas, and (5) slow growth of the human–human hybridomas in nude mice.

Cell lines have been established from patients with multiple myeloma or plasma cell leukemia that secrete monoclonal antibodies identical to the circulating paraproteins (Abrahms *et al.*, 1983; Burk *et al.*, 1978; Edwards *et al.*, 1982; Karpas *et al.*, 1982a,b; Ritts *et al.*, 1983; Togawa *et al.*, 1982; Matsuoka *et al.*, 1967; Nilsson *et al.*, 1970). A variable proportion of the cultured myeloma cells exhibit typical cell plasma morphology, with an eccentric nucleus, condensed nuclear chromatin, prominent Golgi apparatus, and abundant rough endoplasmic reticulum (Kozbor *et al.*, 1983). None-

theless, it is not entirely clear that the cultured cells are mature plasma cells.

Most normal human plasma cells are terminally differentiated. Even in multiple myeloma, the malignant plasma cells usually have a low growth fraction. However, the neoplastic clones in myeloma patients may include a few cells with chracteristics of both lymphocytes and plasma cells. For example, plasma cells generally lack the CR2 receptor for complement, and therefore are not susceptible to infection by Epstein–Barr virus. Our laboratory established a cell line from a patient with multiple myeloma and circulating malignant plasma cells. Thirty to fifty percent of the cultured cells had typical histologic features of mature plasma cells. Repeated subculturing failed to increase this percentage. The immunoglobulin synthesized by the cultured cells was apparently identical to the patient's circulating paraprotein. However, many of the cells contained the Epstein–Barr nuclear antigen (EBNA) as detected by anticomplement immunofluorescence. Samples of other human plasma cell lines sent to our laboratory for EBNA analysis were also antigen positive. EBNA-negative human myeloma cell lines have been produced. However, the rate of immunoglobulin synthesis by the EBNA-negative and positive cell lines does not differ substantially.

Many laboratories have used successfully Epstein–Barr virus-transformed human lymphoblastoid cell lines for somatic cell hybridization and monoclonal antibody production. The Epstein–Barr virus selectively infects and immortalizes human B lymphocytes. Initially, Epstein–Barr virus-transformed B lymphoblasts have a low cloning efficiency and do not form tumors in nude mice. After prolonged propagation *in vitro,* cytogenetic changes that alter the expression of cellular oncogenes may be naturally selected. The final result is a rapid growing cell line with properties very similar to the malignant lymphoblasts from patients with Burkitt's lymphoma.

With repeated cloning, it has been possible to obtain B lymphoblastoid cell lines with a plating efficiency of 50% and a frequency of hybrid formation of 1 in 10^5 cells. Nevertheless, the best human lymphoblastoid cell lines are still not as vigorous as murine myeloma cells. Early after human–human cell fusion, the hybridomas should be cultured over irradiated autologous mononuclear cells, human fibroblasts, or mouse peritoneal cells, to promote clonal outgrowth. Once established, human–human hybridomas will proliferate without feeder cells.

Particularly when EBV-infected cells are used for fusion, it is important to demonstrate that the antibody-producing cell lines are true hybridomas, and not transformed cells from the normal lymphocyte donor. As discussed earlier, one approach to the problem is to use a ouabain (or G418)-resistant cell line as a fusion partner, and to test the hybridomas for growth in HAT–ouabain medium. When this is not available, the DNA content, karyotype, and histocompatibility antigens in the cultured cells should be checked.

B. Human–Human Hybridomas against Bacterial and Viral Antigens

Croce *et al.* (1980a) fused the peripheral blood lymphocytes from a patient with subacute sclerosing panencephalitis with a 6-thioguanine-resistant mutant of the GM-1500 lymphoblastoid cell line (GM-1500 6TG-ALL). After selection in HAT medium, hybridomas were isolated that secreted IgM antibodies against measles virus nucleocapsids. The hybridoma antibodies immunoprecipitated the same viral polypeptides as a convalescent serum from a patient recovering from measles.

Human–human hybridomas secreting antibodies against tetanus toxoid have been generated in several laboratories. This model system has been used because of the ready availability of tetanus toxoid-specific immune B cells from donors immunized *in vivo*. Chiorazzi and co-workers (1982) fused the tonsillar lymphocytes from a tetanus toxoid boosted patient with HAT-sensitive variants of the WI-L2 (WI-L2 AG^R35scl) and GM-0407 lymphoblastoid cell lines (Lever *et al.*, 1974; Levy *et al.*, 1968). One of the hybridomas secreted an IgM antibody against tetanus toxoid. Kozbor *et al.* (1982a) generated an EBV-transformed B cell line that produced an IgM antibody against tetanus toxoid. Antibody secretion was stabilized by fusion with a ouabain-resistant mutant of the GM-1500 6TG-2 cell line (KR-1), followed by selection of hybridomas in HAT–ouabain medium. The hybrids were stable for 1 year and secreted up to 6 μg/ml of an IgM antitetanus toxoid antibody. Larrick and co-workers (1983) selected a 6-thioguanine-resistant lymphoblastoid cell line (LTR228). The LTR228 cells were fused with pokeweed mitogen-activated peripheral blood B lymphocytes, from volunteers vaccinated 9 days earlier with tetanus toxoid. Hybridomas were isolated in azaserine–hypoxanthine medium at an efficiency of 1 in 10^5 cells. Cultures secreting antitetanus toxoid antibody were subcloned in soft agar. Two clones secreted from 1 to 5 μg of specific IgG κ antibody per 10^6 cultured cells over a 24-hour period. The clones remained stable for 11 months in continuous culture. From hybridomas grown in 5-liter spinner flasks, milligram quantities of IgG were purified by Staphylococcus protein A affinity chromatography. The isolated hybridoma antibody protected mice injected with 1000 times the LD_{50} of tetanus toxin.

Freimark and Ozer (1985) isolated a human–human hybridoma secreting an IgG κ antibody, specific for the C fragment of tetanus toxin. The monoclonal immunoglobulin protected mice from a lethal challenge with the toxin (Table III). However, the monoclonal antibody was not as effective as affinity purified human polyclonal antitetanus antibody. The authors concluded that human monoclonal antibodies against different epitopes should be used in

TABLE III
NEUTRALIZATION OF A LETHAL DOSE OF TETANUS TOXIN IN MICE WITH HUMAN MONOCLONAL ANTIBODY[a]

Antitoxin	μg Antibody/mouse	Number dead/number of total at indicated time (hours) after toxin administration					
		24	48	72	96	120	144
Monoclonal antibody	10.0	0/4	0/4	0/4	0/4	0/4	0/4
	1.0	3/4	3/4	3/4	3/4	3/4	3/4
	0.1	4/4	—	—	—	—	—
Polyclonal antibody (No. 1)	10.0	0/4	0/4	0/4	0/4	0/4	0/4
	1.0	0/4	0/4	0/4	0/4	0/4	0/4
	0.1	0/4	0/4	0/4	0/4	0/4	0/4
Polyclonal antibody (No. 2)	10.0	0/4	0/4	0/4	0/4	0/4	0/4
	1.0	0/4	0/4	0/4	0/4	0/4	0/4
	0.1	0/4	0/4	0/4	0/4	0/4	0/4
Control myeloma protein	10.0	4/4	—	—	—	—	—
Buffer control	—	4/4	—	—	—	—	—

[a] All animals received 1.0 ng of tetanus toxin, with or without test antibody, in a total volume of 0.1 ml.

combination, to maximize antibody avidity and lattice formation (Brown, 1982; Ehrlich *et al.*, 1983).

Heitzmann and Cohn (1983) stimulated normal human tonsillar lymphocytes with the B cell mitogen *Staphylococcus aureus* Cowan strain I. The activated cells were fused with the WI-L2-729-HF2 cell line, developed by R. Lundak. After selection in HAT medium over irradiated murine peritoneal cells, a fusion efficiency of one in 10^4–10^5 cells was achieved. One hybridoma secreted an IgG antibody against tetanus toxoid; another reacted with diphtheria toxin. The subcloned hybridomas remained stable for 5 months. Schwaber *et al.* (1984) utilized a similar strategy to prepare human–human hybridomas secreting antipneumococcal antibodies.

Emanuel and co-workers (1984) prepared EBV-transformed lymphoblastoid cells from patients with serologic evidence of recent cytomegalovirus infection. Cultures producing anticytomegalovirus antibody were fused with a secondary mutant of WI-L2-727-HF2-6TG, that was resistant to 10^{-5} *M* ouabain. Hybridomas were cultured over irradiated human fibroblast monolayers. One hybrid cell line produced 5 μg/ml of an IgG κ antibody that reacted with cytomegalovirus-infected cells, as detected by indirect immunofluorescence.

Haemophilus influenzae Type B is a leading cause of serious infectious

disease in children less than 1 year of age. Passive immunization with human immunoglobulin has been suggested as a means of preventing *H. influenzae* infections in high risk infants. As mentioned earlier, a human–mouse heterohybridoma secreting an IgM antibody against *H. influenzae* was isolated by Insel (1984). Similarly, human–human hybridization has yielded monoclonal antibodies against the *H. influenzae* Type B capsular polysaccharide. Hunter and colleagues (1982) selected a mutant human myeloma cell line (HFB-1) that was resistant to 6-thioguanine and secreted little or no immunoglobulin. The HFB-1 cells were fused with splenic lymphocytes from a child with idiopathic thrombocytopenic purpura. One hybridoma clone produced an IgG antibody reactive with the *H. influenzae* capsular polysaccharide. The purified antibody protected suckling rats from the lethal effects of *H. influenzae* infection.

C. Human–Human Hybridomas against Tumor Antigens

The humoral immune response to autologous tumor cells has been difficult to analyze in humans. As discussed earlier, fusion of the lymphocytes infiltrating primary tumors with mouse myelomas has not yielded heterohybridomas sufficiently stable for detailed examination of antigenic specificity. As an alternative approach, human–human B cell fusions have been attempted. Several human hybridomas have been reported to secrete antitumor antibodies, as detected by immunofluorescence. The analysis of these hybridoma-derived monoclonal antibodies is still incomplete, and the results must be evaluated skeptically.

Sikora *et al.* (1982, 1983) isolated lymphocytes from 12 patients undergoing craniotomy for malignant glioma. The cells were fused with an 8-azaguanine-resistant, fast growing mutant of the human ARH-77 myeloma cell line (LICR-LON-HMy2). Seventy-one stable hybridomas were obtained from 5 of the 12 patients. Seven hybridoma supernatants bound weakly to a glioma cell line, and to other tumor cell lines, but not to normal fibroblasts or peripheral blood lymphocytes. One stable hybridoma with glioma-binding activity was expanded in serum-free medium to yield milligram quantities of antibody. The immunoglobulin in the supernatant was purified. After labeling with iodine-131, the hybridoma antibody was injected intravenously into several patients with gliomas. In three subjects, radioactive scanning revealed increased concentration of the radioisotype at tumor sites (Sikora *et al.*, 1983).

Glassy and co-workers (1983a) fused lymphocytes from the regional draining lymph nodes of cancer patients with a HAT-sensitive WI-L2 variant (UC 729-6). One hybridoma produced up to 5 μg/ml of an IgM antibody that reacted with HeLa cells, and with lung and prostate cancer cell lines. As measured by immunofluorescence, the monoclonal antibody did not bind

normal human fibroblasts. Hagiwara and Sato (1984) used the same system to generate a human–human IgM hybridoma antibody against autologous cervical carcinoma cells.

Houghton *et al.* (1983) fused the draining lymph node lymphocytes of patients with malignant melanoma with three human lymphoblastoid cell lines (LICR-LON-HMy2, SKO-007, and GM-1500), as well as with the murine NS-1 myeloma cell line. A total of 158 separate fusions were performed. A panel of 20 human cancer cell lines was used to screen 771 Ig-secreting cultures. One stable IgM monoclonal antibody was secreted by a tetraploid hybridoma derived from fusion of LICR-LON-HMy2 cells with lymphocytes from the axillary lymph nodes of a patient with melanoma. The concentration of immunoglobulin in the culture supernatant reached 5 μg/ml. The antibody bound a glycolipid antigen on the surface of 19 of 61 tumor cell lines. The reactivity of the antibody with normal tissue was not analyzed extensively.

D. Human–Human Hybridomas Producing Autoantibodies

Human–human somatic cell hybridization has successfully generated IgM monoclonal antibodies reactive with DNA, platelets, red blood cells, and IgG. Hybridomas have also been generated that bind uncharacterized cytoskeletal, perinuclear, and nuclear antigens. The studies have provided important information concerning structural relationships among autoantibodies.

In an extensive series of experiments, Shoenfeld and colleagues have analyzed the specificity and idiotypic diversity of hybridoma derived anti-DNA antibodies from patients with systemic lupus erythematosus (Shoenfeld *et al.*, 1982, 1983a,b; Isenberg *et al.*, 1984; Massicotte *et al.*, 1984). Peripheral blood and splenic lymphocytes from patients with active systemic lupus erythematosus, and from patients with cold agglutinin disease, were fused with the GM-4672 variant of the GM-1500 lymphoblastoid cell line. Optimal conditions for obtaining autoantibody secreting hybridomas included the use of 44% polyethylene glycol to promote fusion, a mononuclear cell to GM-4672 cell ratio of 5:1, and prior stimulation of the B lymphocytes with pokeweed mitogen.

Initially, 16 IgM κ autoantibody-producing hybridomas were isolated. Seven reacted with single stranded DNA; six bound to platelets; seven produced cold agglutinins reactive with human lymphocytes. The hybrids were stable for at least 7 months. Immunoglobulin concentrations in the culture supernatants reached 1–15 μg/ml.

The IgM hybridomas were detected initially by binding to DNA-coated plates. However, the autoantibodies were not entirely specific for DNA. Unlike the predominant anti-DNA antibodies in serum, the hybridoma

products reacted with a diverse group of negatively charged, phosphate-containing antigens (Shoenfeld *et al.*, 1983b). Not only nucleic acids, but also cardiolipin, platelet phospholipids, and the cytoskeletal protein vimentin, bound significant amounts of antibody.

Surprisingly, fusion of pokeweed mitogen activated peripheral blood lymphocytes from normal subjects and patients with lupus erythematosus has yielded the same frequency of IgM anti-DNA hybridomas (Rauch *et al.*, 1985; Litman *et al.*, 1983). All the monoclonal autoantibodies were polyspecific, although a few reacted better with native DNA than with denatured DNA. In some patients with rheumatoid arthritis, IgM monoclonal antibodies were obtained that reacted with both DNA and IgG.

Shoenfeld *et al.* (1983a) also evaluated the idiotypic cross-reactions among 60 different polynucleotide-binding monoclonal lupus autoantibodies, produced by human–human hybridization of lymphocytes from seven unrelated patients with systemic lupus erythematosus. Three different rabbit anti-idiotypic antisera, and one murine monoclonal antiidiotypic antibody, detected extensive cross-reactions among the hybridoma autoantibodies. One rabbit antiserum recognized an idiotypic antigen (designated 16/6/R) that was present in increased concentration in the sera of patients with systemic lupus erythematosus.

The idiotypically cross-reactive immunoglobulins in several human–human hybridoma supernatants were purified by anti-IgM affinity chromatography. Sufficient material was obtained to permit partial amino acid sequencing (Atkinson *et al.*, 1985). The light and heavy chains of six idiotypically cross-reactive proteins had nearly identical sequences from residues 1 to 40. The sequences were nearly identical to WEA, a Waldenstrom's IgM that binds *Klebsiella* polysaccharides. The authors concluded that similar light chain variable region structures could be utilized for autoantibodies and for antibodies against environmental pathogens.

In a related series of experiments, Cairns and colleagues (1984) fused GM-4672 cells with tonsillar lymphocytes from a normal donor. Thirteen hybridomas were isolated that produced IgM κ antibodies reactive with single-stranded DNA, as measured by an enzyme-linked immunoabsorbent assay. The hybridomas were stable, thereby permitting an analysis of antibody specificity and cross-reactivity. Again, each IgM anti-DNA antibody was polyspecific, and reacted with DNA, RNA, and cardiolipin.

For uncertain reasons, human–human somatic cell hybridization has not yielded many IgG anti-DNA antibodies, even in patients with active systemic lupus erythematosus. Pokeweed mitogen stimulation of peripheral blood lymphocytes can trigger the *in vitro* synthesis of IgG anti-DNA antibodies. Hence, cells producing IG autoantibodies are not entirely lacking in the blood.

Recently, Jirik *et al.* (1985) fused the peripheral blood lymphocytes of a

patient with chronic lymphatic leukemia, and a monoclonal IgM κ rheumatoid factor, with the WI-L2-729-HF2 lymphoblastoid cell line. More than 50% of the resulting hybridomas produced IgM rheumatoid factor in high titer. Immunoglobulin synthesis was maintained during 8 months propagation in tissue culture. The hybrid nature of the cells was proven by Southern blotting experiments with DNA probes for the κ J region gene segment. Importantly, the hybridoma derived autoantibody shared the same cross-reactive idiotypic determinant with the donor's rheumatoid factor paraprotein.

Human–human hybridomas have recently proven useful in analyzing the immune response of patients with immunologically mediated endocrine diseases. Eisenbarth *et al.* (1982) fused GM-1500-6TG2 lymphoblastoid cells with peripheral blood lymphocytes from patients with diabetes mellitus Type I. An IgM monoclonal antibody was isolated that reacted with human pancreatic islet cells. The hybridoma continued to secrete the autoantibody during 1 year in continuous culture.

Satoh and co-workers (1983) fused the peripheral blood mononuclear cells from four diabetes mellitus patients with the GK-5 variant of GM-1500. The hybridioma supernatants were screened by induced immunofluorescence for binding to human endocrine tissues. Seven hybridiomas produced IgM κ monoclonal antibodies that recognized antigens in the anterior pituitary, thyroid, gastric mucosa, and pacreas. However, the hybridoma antibodies also reacted with intermediate filaments on cultured human and mouse fibroblasts. Dwyer and colleagues (1983) fused the GK-5 mutant lymphoblasts with the peripheral blood cells from a patient with myasthenia gravis. An IgM antibody was obtained that inhibited the binding of a mouse monoclonal antibody against the acetylcholine receptor. The results suggested that the human–human hybridoma technique could be used to immortalize autologous antiidiotypic antibodies. Such reagents could have potential value for *in vivo* immunotherapy.

In a study referred to earlier, Cote and colleagues (1983) prepared an extensive series of human–human hybridomas, by fusion of the lymph nodes or spleens of cancer patients and normal subjects with the LICR-LON-HMy2 or SKO-007 lymphoblastoid cell lines. Among 422 hybridomas from 36 individuals that were analyzed, 9% secreted IgM antibodies reactive with cytoplasmic, perinuclear, and nuclear antigens in normal cells. In contrast, less than 1% of the hybridoma antibodies reacted with antigens on the cell membrane. The specificities of the monoclonal antibodies were not analyzed in detail.

E. Other Antigens

Olsson and Kaplan (1980) sensitized a Hodgkin's disease patient with 2,4-dinitrochlorobenzene. A week later, splenic mononuclear cells were fused

with SKO-007, a HAT-sensitive variant of the U-266 myeloma line. The resulting hybridomas generated up to 11 μg/ml IgG anti-dinitrophenyl antibody. Subsequently, mycoplasma contamination was detected, and successfully treated, in the SKO-007 cells.

Handley and Royston (1982) attempted to immunize human tonsillar lymphocytes *in vitro* with sheep red blood cells. After fusion of the cultured cells with WI-L2-729-HF2 lymphoblasts, anti-sheep erythrocyte antibodies were detected in the culture supernatants. However, the hybridomas were not characterized systematically.

F. Advantages of Human–Human Hybridization

The major advantage of human–human hybridomas is their relative stability. The human parental cell lines are still not ideal. They secrete small amounts of immunoglobulins, contain the Epstein–Barr viral genome in many cases, and require feeder layers for cloning. However, the isolation of hybridomas is relatively straightforward, provided that an enriched source of antigen specific, activated B cells is used for fusion. For unclear reasons, the human–human hybridomas reported to date are predominantly IgM secretors.

VIII. Human × Human–Mouse Heterohybridomas

In an attempt to combine the favorable characteristics of both human and murine parental cell types, human–mouse heterohybridoma cell lines have been rendered suitable for B cell fusion. Teng and colleagues (1983) transfected the HAT-sensitive human FU-266 variant of the U-266 myeloma cell line with the recombinant plasmid vector pSV2-neo^R. The neo^R gene transferred resistance to the neomycin-related antibiotic G-418. The transfected clone was fused with the murine P3-X63-Ag8.653 myeloma. Interspecies hybrids were selected in medium supplemented with G-418 plus ouabain. The murine parent provided the ouabain resistance; the transfected human parent contributed resistance to G-418. Since both parental cells were HPRT deficient, the hybrids retained their HAT sensitivity. Subsequently, a subclone was selected that secreted little or no human immunoglobulin. The heteromyeloma mutant was used as a fusion partner for human lymphocytes from several sources, including peripheral blood, spleen, and lymph nodes. The heterohybridomas were superior to the parental mouse myeloma cell line in test fusions. Heterohybridomas were isolated that bound the 2,4-dinitrophenyl hapten, tetanus toxoid, DNA, RNA, and Rh factor.

In a follow-up study, Teng *et al.* (1985) immunized two Hodgkin's disease patients with a killed *Escherichia coli* 011:B4 vaccine. One week later, the surgically removed splenic cells were fused with the G418-ouabain cross-

resistant human–mouse heteromyeloma. Alternatively, the splenic B cells were first infected with EBV, and were fused 14 days later. In both cases, heterohybridomas emerged that secreted antibodies against *E. coli* endotoxin. Ninety percent of the clones were IgM producers. The heterohybrids produced 2–30 μg/ml IgM in a 24-hour period. One clone was studied in detail. It secreted an IgM antibody reactive with lipopolysaccharides from several different species of gram-negative bacteria. Importantly, the heterohybridoma-derived monoclonal antibody successfully protected mice from lethal gram-negative bacteremia.

Foung and co-workers (1984) fused the SP-2 murine myeloma cell line with normal human peripheral blood B lymphocytes. A heterohybridoma with stable human immunoglobulin production was recloned in the presence of 6-thioguanine. The resulting HAT-sensitive, ouabain-resistant variant was fused with an Epstein–Barr virus-transformed human splenic lymphoblastoid cell line that secreted IgM antibody against erythrocyte group A antigen. The human X (human × mouse) heterohybridomas were cloned over mouse fibroblast feeder layers. One cell line produced IgM anti-A antibody at a higher titer than the parental human lymphoblastoid line (about 3–4 μg/24 hours/10^6 cells). The authors reported that the same technique had yielded human monoclonal antibodies reactive with varicella-zoster and with Epstein–Barr viral antigens. Osberg and Pursch (1983) used the same strategy to produce human X (mouse × human) hybridomas stably producing human antibodies.

In principle, the human–mouse heteromyelomas should be ideal partners for the generation of human monoclonal antibodies. However, based upon experiments published to date, and the personal experience of the authors, it is still not clear that the human–murine heteromyelomas are superior to the available human and murine cell lines.

IX. Characterization of Human Monoclonal Antibodies

The characterization of human monoclonal antibodies produced by human–human and human–mouse hybridomas, as well as by EBV transformed B cells, has often been inadequate. The biological significance of antibodies reactive with autologous tumors and self antigens depends crucially upon a thorough analysis of antibody specificity. IgM antibodies will bind multivalently to many antigens with repeating determinants. Aggregation of proteins or nucleic acids on a solid phase enhances such weak interactions. These considerations could explain the high percentage of IgM-secreting hybridomas and EBV-transformed cell lines that react with DNA, vimentin, and perhaps aggregated IgG. It has been estimated that any given monoclonal antibody could react with 1 in 20 antigens with an affinity of 10^3

M^{-1}, and 1 in 200 antigens with an affinity of 10^5 M^{-1} (Brown, 1982). Thus, a yield of 0.5–1% of IgM-secreting hybridomas that bind either autologous tumor tissue, or autoantigens, is not unexpected. On a practical level, antibodies of such low affinity would prove difficult to use for drug delivery, diagnostic imaging, or idiotypic modulation.

The possibility also remains that even high affinity antibodies captured and immortalized by somatic cell hybridization are not representative of the antibodies produced by the patient *in vivo*. Human–human and human–mouse somatic cell hybridization is an extremely inefficient process. In both systems, typical fusion frequencies are in the order of 1 in 10^5 cells. Perhaps the donor B cells that yield successful heterokaryons derive predominantly from a skewed subpopulation of IgM secretors, that are relatively inactive *in vivo*. Particularly with human–human hybrids, one regularly obtains scrambled immunoglobulin molecules formed by the combinatorial association of heavy and light chains from the normal donor lymphocytes and the hybridization partner. It is certainly conceivable that these hybrid molecules could display novel antibody specifities generated during the fusion process.

X. Propagation of Human–Mouse and Human–Human Hybridomas *in Vivo*

It is our experience as well as others that the majority of antigen-specific human–mouse and human–human hybridoma cell lines accumulate only a few micrograms of immunoglobulin per milliliter of spent tissue culture medium. To obtain milligram quantities of antibody it is necessary to produce large volumes of tissue culture supernatant. Methods have been developed which allow high cell densities to be reached in tissue culture (Table IV). These include the use of a cytostat for feeding and harvesting (Fazekas de St. Goth, 1983; Feder and Tolbert, 1983) and the growth of cells in hollow fibers (Netzer, 1983) and encapsulated in beads (Klausner, 1983). Although these techniques entail considerable expense, they overcome the low cell density achieved in static tissue culture.

With murine hybridomas grown in normal mice as ascites tumors immunoglobulin concentrations in ascitic fluids can reach 5–10 mg/ml. In contrast, both human–mouse and human–human hybridomas must be propagated in immunodeficient mice to avoid xenograft rejection. Abrams *et al.* (1984) rescued immunoglobulin secretion from two chronic lymphatic leukemia cell populations by fusion with the murine NS-1 cell line. Pristane primed athymic nu/nu mice each received 10 million heterohybridoma cells. Ascites developed after 3 weeks. The concentration of human IgM in the ascitic fluid reached 1.7 mg/ml. Insel (1984) similarly passaged heterohybridomas producing an IgG antibody against *H. influenzae* type b in pristane primed athymic nude mice. A maximum of 29 mg human immu-

TABLE IV
LARGE QUANTITY ANTIBODY PRODUCTION FROM HUMAN HYBRIDOMAS

Source	Quantity	Expense	Reference
Tissue culture supernatants	0.2–20 μg/ml	Low	Many authors
Nude mouse ascites	0.5–2.0 mg/ml	Moderate	Abrams *et al.* (1983); Truitt *et al.* (1984); Freimark (1985)
Cytostat	Milligrams/grams	High	Fazekas de St. Goth (1983); Feder and Tolbert (1983)
Hollow fibers	Milligrams/grams	High	Netzer (1983)
Encapsulated in beads	Milligrams/grams	High	Klausner (1983)

noglobulin was obtained from a single mouse. Freimark (1985) successfully propagated in mice a human–human monoclonal antitetanus toxoid antibody. Nude mice primed with pristane 1 week prior to intraperitoneal injection of twenty million hybrid cells failed to develop ascites. However, if mice were given 400 rads of X-irradiation 1 week prior to hybrid injection, 25–50% of the mice developed ascites in 2–4 weeks. The acitic fluid from two different cloned hybrids contained up to 1.2 mg/ml human immunoglobulin, compared to 2 μg/ml in culture supernatants (Fig. 1). Truitt *et al.* (1984),

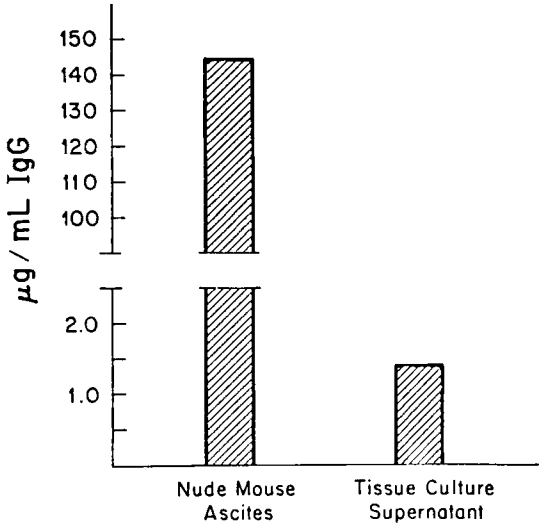

FIG. 1. Yield of human antitetanus toxoid monoclonal antibody in tissue culture supernatant versus nude mouse ascites. Hybrid cells were either inoculated into irradiated-pristane primed nude mice, or were grown in spinner flasks. Ascites and tissue culture supernatants were assayed for human IgG by enzyme-linked immunosorbent assay.

also found that human–human hybridomas will only grow in pristane primed irradiated nude mice. In their system, subcutaneous adaptation of the hybridomas was necessary prior to intraperitoneal inoculation.

XI. Genetically Engineered Antibodies

The production of monoclonal antibody by the transfection and expression of cloned immunoglobulin genes is now feasible. To date, nearly all experiments have utilized murine immunoglobulin genes, and are beyond the scope of this review (Ochi *et al.*, 1983a,b; Oi *et al.*, 1983; Deans *et al.*, 1984). However, recombinant DNA techniques are equally applicable to human monoclonal antibody systems. Indeed, the creation of mouse–human chimeric antibodies has recently been achieved.

The isolation of stable "transfectomas" expressing murine immunoglobulin genes has been accomplished with appropriate vectors containing a functional mammalian transcription unit. These included (1) coding sequences for immunoglobulin light or heavy chains, (2) a selectable bacterial gene such as neo^R or *E. coli*-gpt, and (3) viral or mammalian promoter, splice, and polyadenylation sites. Additionally, the vectors contain the pBR322 *ori* and β-lactamase genes to permit the propagation and selection of the plasmids in *E. coli* (Morrison and Oi, 1984). Vectors based upon SV40, Epstein–Barr virus (Sugden *et al.*, 1985), and defective amphotropic retroviruses (Sorge *et al.*, 1984; Williams *et al.*, 1984) have been used successfully to transfer genes into human B lymphocytes and bone marrow cells.

Although all human cells contain immunoglobulin genes, the synthesis and secretion of intact antibodies are confined to B-lymphocytes and plasma cells. In mouse systems, only B or plasma cells have produced intact antibody molecules after transfection of immunoglobulin genes. The transfer and expression of human immunoglobulin light or heavy chain genes into B lymphoblasts have not yet been reported.

Mouse immunoglobulin light and heavy chain genes have been introduced into *E. coli* using expression vectors (Boss *et al.*, 1984). The bacteria synthesized small amounts of immunoglobulin polypeptides. The light and heavy chains were not glycosylated by the bacteria and were therefore relatively insoluble. Kenten *et al.* (1984) coupled a fragment of the cloned gene for the human ϵ chain to the tryptophan control region of an expression plasmid, and introduced the recombinant DNA into *E. coli*. An antigenically active polypeptide of 40,000 MW constituted 18% of total bacterial protein. The nonglycosylated ϵ chain fragment was aggregated and packed into large inclusion bodies within the bacterial cell. After extraction into denaturing solvents, and purification by antibody affinity chromatography, monomeric

and dimeric epsilon chains were isolated. The cloned gene product inhibited the binding of human IgE to cultured basophils.

In a widely publicized experiment, Morrison and co-workers (1984; Morrison and Oi, 1984) created mouse–human chimeric antibody molecules by taking the variable region genes of a mouse myeloma and joining them to human immunoglobulin constant region genes. The murine S107 myeloma cell line produces an IgA κ anti-phosphorylcholine antibody. The rearranged, cloned S107 κ variable region exon was spliced to a human κ gene at a site in the intron between the joining and constant region exons. The chimeric gene was inserted into the pSV2-neo plasmid vector. Similarly, the S107 heavy chain variable region gene was spliced to human IgG_1 and IgG_2 constant region genes, and was inserted into the pSV2 ΔH-gpt vector.

Both chimeric genes were transfected into murine myeloma cells, using the protoplast fusion or calcium phosphate precipitation techniques. The double transfectants were isolated in selective medium containing mycophenolic acid, xanthine, and G418. Among 70 cell clones analyzed, 7 were positive for human immunoglobulin by radioimmunoassay. Two produced significant quantities of both heavy and light chains. Importantly, the immunoglobulin molecules were glycosylated appropriately. The transfected cells were propagated successfully in BALB/c mice. Analyses of sera and ascitic fluids revealed anti-phosphorylcholine antibodies with human heavy and light chain constant region determinants.

Chimeric mouse–human immunoglobulin gene constructs permit the creation of "near human" antibodies with predefined antigen-binding specificities. It has been proposed that such antibodies will be less immunogenic in humans than mouse monoclonal antibodies. Clinical trials will be necessary to test this hypothesis.

The merging of gene cloning and human hybridoma technology can provide novel information concerning (1) structure–function relationships among immunoglobulins, (2) the immunoglobulin gene sequences that give rise to autoantibodies, and (3) the molecular basis of B cell immunodeficiency syndromes. On a practical side, the transfection of cloned human immunoglobulin genes into murine myeloma cells may provide an efficient means for stabilizing antibody secretion. Eventually, it should be possible to create "genetically immunized" animals, in which human immunoglobulin genes coding for desired antibodies have been inserted into the germ line (Grosschedl *et al.*, 1984; Storb *et al.*, 1984).

Conceivably, DNA transfection may be used to immortalize human B lymphocytes and plasma cells, as an alternative to somatic cell hybridization and EBV transformation. A number of transforming B cell-specific oncogenes have recently been described (Morton *et al.*, 1984). Jonak *et al.* (1984) transfected murine spleen cells with DNA from the human leukemia

cell line Reh, and obtained permanent antibody-producing plasma cell lines. Human monocyte–macrophage cell lines have also been generated by DNA transfection. In principle, the same strategy should be applicable to human B lymphocytes and plasma cells.

XII. Concluding Remarks

Methodologic difficulties have hindered the production and dissemination of human monoclonal antibodies. Antigen-specific human B lymphocytes or plasma cells are seldom available in quantity. Techniques for B cell immortalization by EBV transformation, and somatic cell hybridization, are inefficient and may select minor lymphocyte subpopulations. Hybridomas producing human monoclonal antibodies frequently are not stable with time. The bulk preparation of human monoclonal antibodies requires costly cell culture or passage of xenogenic cell lines through immunodeficient mice.

Gradually, these problems are being overcome. Current targets of research include (1) the isolation of rare antigen specific B cells and their expansion in media containing purified B cell growth factors, (2) the specific joining of cells with antibodies and electric fields to increase hybridization frequencies, (3) the generation of nonsecreting human plasma cell lines, or murine myeloma cell lines with drug markers that prevent the loss of heavy and light chain genes, (4) the creation of monoclonal antibody-secreting cell lines by DNA transfection, and (5) the stabilization and large scale production of human monoclonal antibodies by the expression of human immunoglobulin genes in murine myeloma cells and transgenic mice.

Once perfected, human monoclonal antibody technology will make possible a detailed analysis of the human humoral immune response on a clonal level. The specificity and diversity of autoimmune and allergic responses, and their regulation by immune networks, will be elucidated. Conflicting data concerning the role of humoral immunity in the control of tumor growth and metastasis will be brought into focus.

Preliminary experiments suggest that the *in vivo* administration of human monoclonal antibodies lacks acute toxicity. The long-term safety of human monoclonal antibodies can only be ascertained by prospective clinical trials with appropriate precautions. The therapeutic potential of human monoclonal antibodies has not yet been realized. Most investigators believe that human monoclonal antibodies will be preferable to murine immunoglobulins for drug delivery and *in vivo* immune modulation.

ACKNOWLEDGMENTS

Research in the authors' laboratory is supported by National Institutes of Health Grants AM25443, AM07144, and RR00833. We thank Marla Spierman and Jane Uhle for editorial assistance.

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ADVANCES IN IMMUNOLOGY, VOL. 38

Maternally Transmitted Antigen

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I. Introduction

Maternally transmitted antigen (Mta) is an unusual polymorphic mouse cell surface antigen present on normal T and B cells and on cultured cell lines of diverse tissue origin (Fischer-Lindahl *et al.*, 1980). It is defined by cytotoxic T lymphocyte (CTL) recognition; to date no antibodies to Mta have been reported. Mta disparity also induces skin graft rejection as well as weak secondary mixed lymphocyte responses (Chan and Fischer-Lindahl, 1985). The determinant is associated intimately with a molecule which exhibits important properties of class I major histocompatibility antigens; its expression is modified by a locus, *Hmt*, linked to the *Tla* region of the *H-2*

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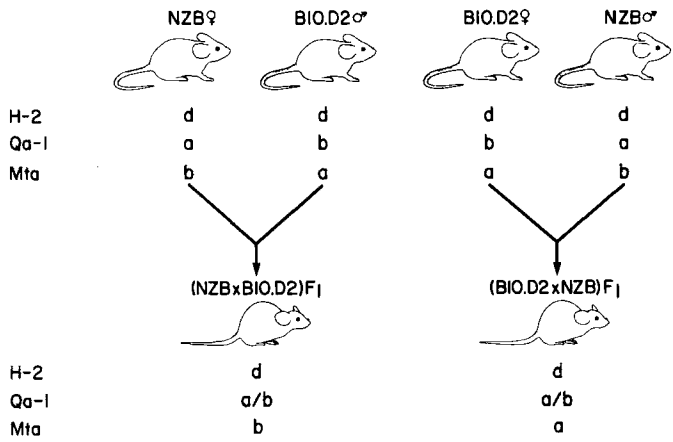

FIG. 1. Maternal inheritance of Mta. The inheritance pattern of Mta is illustrated by the paradigmatic reciprocal crosses of B10.D2 (K^d, D^d, Qa-1^a, Mta^a) with NZB/B1NJ (K^d, D^d, Qa-1^a, Mta^b). In the cross illustrated on the left an NZB dam mated with B10.D2 transmits both Type I mitochondrial DNA (not shown) and Mtf^β to her progeny; the F_1 progeny are Mta^b. In the reciprocal cross (right), a B10.D2 female transmits Type V mtDNA and Mtf^α to the F_1 progeny.

complex (Fischer-Lindahl *et al.*, 1983). Mta polymorphism itself, however, is determined by an element, *Mtf*, that is transmitted maternally. That is, F_1 offspring from a mating between an Mta^a female and an Mta^b male always express Mta^a, while F_1 offspring from the reciprocal cross of Mta^b female with Mta^a male always express Mta^b (Fig. 1). Restriction enzyme polymorphism analyses, and transfer of mitochondria via cell fusion indicate that the genetic element controlling the Mta polymorphism is probably located within mitochondria (Smith *et al.*, 1983; Huston *et al.*, 1985). In this review we summarize the evidence regarding the genetic mechanisms of Mta transmission and the class I nature of the surface antigen. In addition, we will raise certain questions regarding the cell biological, evolutionary, and immunological implications of Mta, and outline areas we believe will be fruitful for future investigations.

The mechanism of transmission alone places Mta in an unprecedented class and raises profound questions concerning cell and evolutionary biology. For example, how could genes located within mitochondria be expressed phenotypically on the cell surface? We will refer to this as the "topological problem." Mechanisms are recognized for export of proteins through the cytoplasmic membrane (Walter *et al.*, 1984), or for import into the mitochondria through two membranes (Hurt *et al.*, 1984; Hase *et al.*, 1984); there is no conclusive evidence suggesting a mechanism for export of mitochondrially encoded proteins (e.g., Beattie, 1971; Neupert and Ludwig,

1971; Alberts *et al.*, 1983). On the other hand, scattered and unconfirmed reports have suggested mitochondrial protein export, or membrane fusions between mitochondria and RER (Klee and Sokoloff, 1965; Bucher, 1967; Ruby *et al.*, 1969; Cohen, 1970; Bracker and Grove, 1971; Franke and Kartenbeck, 1971; Morre *et al.*, 1971; Tipton *et al.*, 1973; Macklin, 1977; Shore and Tata, 1977; Meier *et al.*, 1978; Spacek and Lieberman, 1980; Montisano *et al.*, 1982). In addition, the well-known system of lysosomal antigen processing (Unanue, 1984) might be capable of transporting mitochondrial peptides to the cell surface. Thus, although the actual demonstration of mitochondrial protein export would be unprecedented, the "topological" problem is empiric rather than logical. Indeed, on the basis of the theory of endosymbiotic origin of eukaryotic organelles (Margulis, 1970; Dodson, 1979) one should not be surprised that modern mitochondria would retain the protein export capacities of their free-living ancestors.

The non-Mendelian inheritance of surface antigens is a venerable problem. In particular, the related phenomena of *antigen variation* (phase variation) and *phenotype switching* are relevant to this discussion. Among these are instances of non-Mendelian inheritance of *killer* phenotype in *Paramecium* (Preer, 1971), immobilization antigens (i-antigens) in *Paramecium*, and mating type in both *Paramecium* and *Tetrahymena* (Sonneborn, 1977). These systems are instructive. The first of these is due to a complex intracellular ecosystem of bacterial and bacteriophage endosymbionts. Uniparental inheritance of i-antigens and i-antigen switching were originally thought to reflect cytoplasmic inheritance (the "plasmagene hypothesis"—formulated before mitochondria were known to carry genetic information). Although additional factors have not been excluded formally, these phenomena, as well as mating type switching, are probably dependent on the unusual properties of organisms containing both macronuclei and micronuclei (Sonneborn, 1977; Preer, personal communication). The history of i-antigen studies provides a sobering caveat regarding attempts to implicate extranuclear genetic elements in the expression of surface antigens.

Recognition of Mta by CTL is apparently not *H-2* restricted; this observation raises particularly important immunological questions. The most obvious explanation for this observation, particularly in view of both the genetic mapping of *Hmt* to the MHC, as well as the probable association with β_2-microglobulin (Section IV), is that the Mta determinant(s) represents a specific modification of a monomorphic class I or class I-like molecule. Alternatively, the determinant recognized by Mta-specific CTL may be a conventional antigen seen in the context of a monomorphic restricting molecule. The former interpretation suggests a specific function for a monomorphic class I-like antigen in communicating maternally transmitted genetic information to the cell surface. The latter interpretation raises the prob-

lem of why the putative conventional antigen is not also restricted by the K and D(L) class I antigens, in the context of which CTL recognize other conventional antigens.

II. Nomenclature

The Antigen. Mta (maternally transmitted antigen) refers to a hypothetical molecule or molecules bearing determinants recognized by specific cytotoxic lymphocytes (CTL). As yet no physical or biochemical evidence attests to the actual existence of a particular molecular form of Mta. Three principal forms of Mta have been identified and are designated with Roman superscripts: Mta^a, Mta^b, and Mta^c. These have the strain distribution indicated in Table I. "Mta" will also refer to the phenotype of a cell or strain that expresses Mta surface antigen.

In discussions of a model of possible nuclear inheritance of a maternal effect (Section VI), *Mta* will refer to a hypothetical regulatory nuclear gene; otherwise we will never refer to "Mta" as anything other than the antigen or phenotype.

Determinants. CTL define clonotypically at least one determinant on each of the antigen forms Mta^a and Mta^b. The determinants are designated with "Mta," followed by a decimal point and a number corresponding to a unique determinant. Thus, the determinant(s) recognized on Mta^a-bearing cells by CTL generated by an Mta^b anti-Mta^a response is defined as Mta.1 (Fig. 2). The determinant(s) recognized by Mta^a anti-Mta^b CTL is defined as Mta.2.

Hmt (*h*istocompatibility, dependent on a *m*aternally *t*ransmitted factor) is an *H-2*-linked, Mendelian locus controlling expression of *Mta*.

Mtf (*m*aternally *t*ransmitted *f*actor) refers to the non-Mendelian element or factor determining which variant form of Mta is expressed. Thus Mtf^{α}, Mtf^{β}, and Mtf^{γ} determine expression of Mta^a, Mta^b, and Mta^c, respectively.

Strain Designations. Reference to "NZB" substrains will imply those strains of NZB which express Mta^b (Table I) unless the context clearly indicates otherwise.

Species. We will use current taxonomic schemes that place laboratory strains of mice as well as certain western European wild mice in the species *Mus domesticus*. *Mus musculus* and its subspecies (*M. m. castaneus*, etc.) constitute a closely related but separate taxon (Ferris *et al.*, 1983b).

Crosses. By convention the maternal strain is listed first in crosses: thus (NZB × BALB/c)F_1 refers to offspring of an NZB dam and a BALB/c sire.

Mitochondrial DNA (mtDNA). Diagnostic restriction enzyme digestions are used to classify mtDNA from different strains of mice. The Roman numeral designations used (Table I) are those of Ferris *et al.* (1983a). The major differences between Type I (NZB) mtDNA and others are indicated in Fig. 3.

TABLE I

STRAIN DISTRIBUTION OF Mta VARIANTS

Cytoplasmic variants	
mtDNA type	Species, subspecies, and strains[a]
Mta[a] strains (*Mtf*$^{\alpha}$)	
II[b]	SF/Cam/J
III	IS/Cam/J
IV	NMRI/Navy
V	AKR/J, AU/SsJ, BALB/cJ, CBA/J, CE/J, C3H/HeJ, C57BL/6J, C57BL/10, C58/J, DBA/2, NMRI/Lac, NZB/Full, NZB/Ibm, NZC/OU, NZC/Fgu, NZW/Ola, PL/J, RIIIS/J, SJL/J, SWR, 129
V[c]	A.AL, A.CA, BALB.B, BALB.K, BALB/cByJ, BDP/J, BUB/BnJ, B6.AK1, B6-H-2^{k}, B6.K1, B6.K2, B6-Tla^{a}, B10.A(4R), B10.A(5R), B10.BR, B10.CAS1, B10.D2, B10.DRB62, B10.KPA42, B10.KPA44, B10.M, B10MO11, B10.RIII(71NS), B10.S, B10.WB, B10.Y, C3H/Tif, C3H-H-2^{o}, C3H.JK, C3H.Q, C3H.Pgk-1, C3H.SW, C57L/J, DDK, HtI, I/LNJ, I/LnReJ, I/St, ICR/Swiss, LG/J, LP/J, MA/MyJ, NFS/N, NIH/Ola, NZB/B1Pt, NZW/CrBom, NZW/OU, P/J, SEA/GnJ, SEC/1ReJ, SM/J, ST/bJ, STU, WB/ReJ
VI	Mil II
VII	Zadar
VIII	Peru
IX	Hov, Cittaducale
Untyped mtDNA	*M. musculus* (Denmark), *M. domesticus* (U.K., Isle of May, Sweden, France, Italy, Yugoslavia, Israel, United States, Peru), *M. m. molossinus* (Japan), CAST/Ei (*M. m. castaneus*), *M. m. castaneus* (Thailand), *M. spretus* (Portugal), *M. m. bactrianus*
Mta[b] strains (*Mtf*$^{\beta}$)	
I	NMRI/Bom, NZB/B1NJ, NZO/Dus, NZB/Bom
I[c]	NMRI/Ico, NMRI/Han, NZB/Hz, NZB/Icr, NZB/Ola, NZB/OU, NZO/OU, Rb(6.12)3sic
Mta[c] strains (*Mtf*$^{\gamma}$)	
Unique	WLA76 (Toulouse), BFM/2, (Montpellier), wild *M. domesticus* from Milano and Pavia

Nuclear variants	
Hmt allele	Strains or species
b(null)	*M. m. castaneus*, B10.CAS2
(null)	*M. domesticus* (wild from Milano)
c	*M. spretus* (four stocks), *M. m. bactrianus* (BAC-LAH stock)

[a] All of these strains have been typed with Mta-specific CTL reagents. This listing is based on Fischer-Lindahl *et al.* (1980, 1983), Fischer-Lindahl and Hausmann (1983), and Huston *et al.* (1983).

[b] Ferris *et al.* (1983), and Huston *et al.* (1983).

[c] mtDNA type is inferred from strain source. Mta type was determined by CTL reactivity.

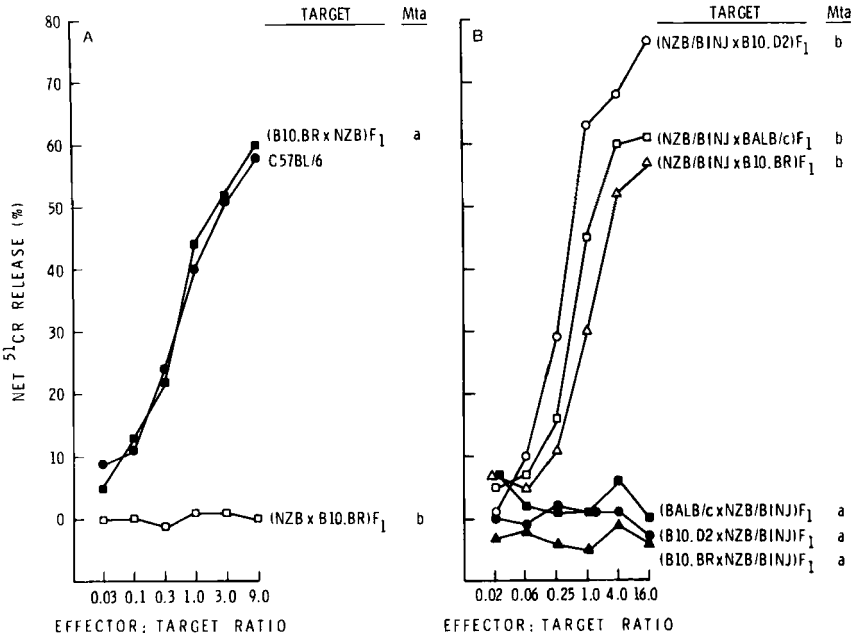

FIG. 2. Typing of mouse lymphoblasts with cloned CTL specific for Mta. Mta-specific CTL lines were tested for their ability to lyse targets in a standard 4 hour ^{51}Cr release assay. Targets: open symbols, Type I mtDNA; closed symbols, Type V mtDNA. (A) The Mta^a-specific CTL line 5F3 [derived from (NZB/BINJ×B10.D2) anti-BALB/c secondary MLC]. (B) The Mta^b-specific CTL clone 4A6, derived from a (B10.BR×NZB/BINJ) anti-NZB primary *in vitro* MLC. Data from Smith and Rich (1985).

H-2 and major histocompatibility complex (MHC) will refer both to the traditional mouse MHC (encoding the class I antigens K, D, and L, and class II antigens), as well as the more distal region of mouse chromosome 17 encoding the class I antigens Qa-2, Qa-1, TL, and others (reviewed by Flaherty, 1984; Flaherty and Lynes, 1984; Rich and Cook, 1984). As will be discussed in a later section, this definition no longer permits a simple distinction between the *MHC* and the *T/t* complex, as the two complexes are interwoven in both wild-type and mutant haplotypes (Artzt, 1984; Shin *et al.*, 1984; see Fig. 5).

III. Maternal Inheritance of Mta Polymorphisms

By far the most intriguing aspect of Mta is its strict maternal inheritance. We are unaware of any other *cell surface antigen* in metazoa that is inherited in this manner. Although the conclusive experiments have yet to be com-

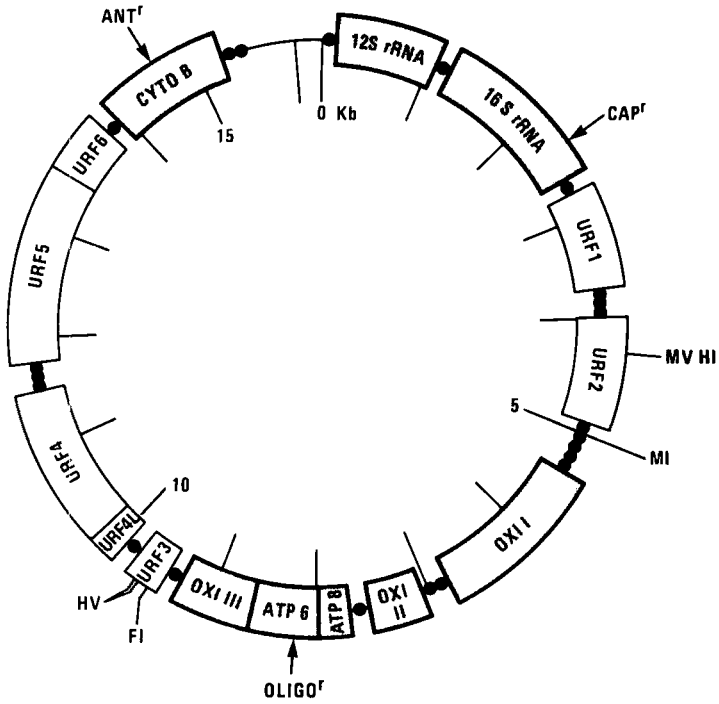

FIG. 3. Map of mouse mitochondrial DNA (mtDNA). The physical location of mitochondrial genes on the 16.3 kilobase pair circular genome. The entire sequence of Type V mtDNA is known (Bibb *et al.*, 1981). Shaded areas: structural genes encoding identified functions (*OXI*, cytochrome oxidase subunits; *ATP*, ATPase subunits; *CYTO B*, cytochrome *B*); open areas: structural genes encoding expressed proteins of unknown function (*URF*s; Michael *et al.*, 1985). Position O is defined at the origin of replication of the heavy strand. Solid circles: tRNAs. The position of useful restriction enzymes sites that distinguish Type I (NZB) from Type V (most laboratory strains) mtDNA: M, *Mbo*I; H, *Hin*fI; F, *Fnu*DII. The *Mbo* site at approximately 5 kb distinguishes Type I mtDNA from 17 other strains. Also shown are the approximate locations of several antibiotic resistance markers available in mammalian mtDNA: *CAP*, chloramphenicol; *OLIGO*, oligomycin; *ANT*, antimycin.

pleted, we think that the evidence for mitochondrial control of Mta phenotype is rather persuasive. We are also unaware of any other case of a surface antigen controlled by mitochondrial (or chloroplast) genetic elements in any eukaryote. The observations by Wilkie *et al.* (1983) that mitochondrial DNA damage can result in modifications of numerous surface features in yeast are intriguing in this respect (John, 1984); however, these modifications could well represent induced modifications of nuclearly encoded surface features.

Furthermore, although polymorphisms of mitochondria are easy to find in

nature or under experimental conditions in lower eukaryotes such as yeasts (see Dujon, 1983, for a review) and fungi (e.g., *Neurospora, ibid;* senescence in *Podospora,* Vierny *et al.*, 1982; Esser *et al.*, 1983), few functional polymorphisms are known in higher eukaryotes (reviewed in Gillham, 1978). Mitochondrial DNA rearrangements and mitochondrial plasmids are strongly implicated in the mechanism of *cytoplasmic male sterility* and susceptibility to Southern corn blight in maize (Laughnan and Gabay-Laughnan, 1983); such involvement may be widespread among higher plants (Edwardson, 1956, 1970). It has been impossible so far to demonstrate conclusively that mitochondria are the causal agents in this phenomenon, due in part to the difficulty of studying somatic cell genetics in higher plants. Suggestions that mitochondrial inheritance underlies variable susceptibility to teratogens in mouse strains (Goldstein *et al.*, 1963; Verrusio *et al.*, 1968; reviewed by Biddle and Fraser, 1977; Morriss, 1979) and certain human disorders (Lynch and Guirgis, 1973; Kodama *et al.*, 1979; Cagianut *et al.*, 1981; Egger and Wilson, 1983; Myers *et al.*, 1983) are intriguing but remain unconfirmed; no studies of mtDNA have as yet been reported.

The discovery that Mta is maternally inherited was, in part, serendipitous. In the late 1970s several groups were engaged in studies of alloantigens mapping to the right of *H-2D* that had certain properties similar to the class I antigens K and D (Stanton and Boyse, 1976; Flaherty, 1976; Wernet and Klein, 1979; Fischer-Lindahl and Hausmann, 1980; Kastner and Rich, 1979; Kastner *et al.*, 1979a, b; Klein and Chiang, 1978; reviewed by Yokoyama *et al.*, 1983; Boyse, 1984; Fischer-Lindahl and Langhorne, 1981; Flaherty, 1981; and see the following). For example, NZB strains and BALB/c (both $H\text{-}2^{d}$) differed at a locus termed *Qa-1* (reviewed by Flaherty, 1984; Flaherty and Lynes, 1984; Rich and Cook, 1984). In particular these antigens stimulated CTL responses that were not restricted by the traditional *H-2* antigens in the *K/D* region.

Fischer-Lindahl and her colleagues (1980) observed that when NZB/BlNJ ($H\text{-}2^{d}$, $Qa\text{-}1^{a}$) spleen cells received primary immunization *in vivo* with BALB/c ($H\text{-}2^{d}$, $Qa\text{-}1^{b}$) stimulators, followed by secondary stimulation *in vitro,* the MLC cultures could lyse targets from most other strains, irrespective of their *H-2* or *Qa-1* type. Thus, these cultures lysed not only BALB/c cells but also BALB.B ($H\text{-}2^{b}$, $Qa\text{-}1^{b}$) and B6.Tla^{a} ($H\text{-}2^{b}$, $Qa\text{-}1^{a}$). These cultures also lysed targets from certain other NZB strains, such as NZB/Füll and NZB/B1Pt (from which the former was derived). Only a few strains were insensitive to lysis by these CTL: NZB/B1NJ itself, a few other NZB substrains, and some but not all mice of the outbred NMRI strains (see Table I).

The secondary CML response of NZB anti-BALB/c thus defined a typing reagent that identified a new alloantigen, subsequently termed "Mta," with an unusual strain distribution. Most strains were typed Mta^{+}, while NZB/B1NJ and similar strains were "Mta^{-}." Because positive typing reagents are

now available to identify an Mta antigen on "Mta$^-$" cells (see Fig. 2), we will use the term Mta^b to refer to these "Mta$^-$" strains.

Fischer-Lindahl and co-workers (1980) decided to map Mta genetically using a series of recombinant inbred lines (Riblet *et al.*, 1980) derived from NZB/Icr (H-2^d, Qa-1^a, Mta^b) and C58/J (H-2^k, Qa-1^a, Mta^a). These lines, founded from F_2 animals and inbred for 20 generations of sister–brother matings, differed with respect to Mta expression. In these lines, Mta was not linked to *H-2* nor any other available chromosomal markers, including the X chromosome. A strict concordance was observed, however, between Mta and the maternal lineage of the recombinant inbred line. All lines tracing maternal lineage to NZB/Icr were Mta^b; all lines tracing maternal lineage to C58/J were Mta^a. The maternal mode of inheritance was immediately confirmed using reciprocal F_1 animals (Fig. 1). F_1 hybrids from an (NZB×BALB/c) mating were Mta^b; F_1 progeny from the reciprocal (BALB/c×NZB) mating were Mta^a. The maternal inheritance of Mta in reciprocal F_1 mice in this and other strain combinations has been confirmed repeatedly (Fischer-Lindahl *et al.*, 1980, 1983; Fischer-Lindahl and Hausmann, 1983; Smith *et al.*, 1982, 1983; Huston *et al.*, 1983). Backcrosses of F_1 females to males of the paternal strain yield backcross mice which express the maternal phenotype. Indeed, 11 generations of such backcrossing exhibited exclusive maternal inheritance of the phenotype (Fischer-Lindahl and Hausmann, 1983).

These results leave little doubt that Mta is controlled by a non-Mendelian factor, inherited through the mother, and not through the father. This factor has been termed *Mtf* (*m*aternally *t*ransmitted *f*actor; Fischer-Lindahl *et al.*, 1983).

Maternal Effect or Maternal Transmission? Maternally influenced non-Mendelian hereditary mechanisms may be loosely classified as *maternal effects* and *maternal transmission* (see discussions by Waddington, 1939; McLaren, 1962, 1976, 1979; Wright, 1968; Grun, 1976; and the volume edited by Newth and Balls, 1979). The former include intrauterine or intra-ovum influences on egg and embryonic development. In general, maternal effects may be interpreted as extensions of the maternal phenotype into the next generation, and are limited by the extent to which the progeny are dependent on maternal processes for nourishment, protection, etc. Therefore, *maternal effects are typically exerted on the immediate offspring only or become diluted over several generations of backcrossing to the paternal strain.* Maternal effects are not determined by actual transmission of genetic material, but often by maternal deposition of a factor during öogenesis or embryogenesis. Hence maternal effects often govern the fertility and fecundity of interspecific hybridization, embryonic development, embryo lethals, susceptibility to teratogenic agents, etc.

In contrast, *maternal transmission* involves the differential transmission of

some form of genetic material such as viruses and other parasites, or cytoplasmic organelles (chloroplasts and mitochondria). *The expression of a maternally transmitted trait is usually stable over many generations of backcrossing females to the paternal strain.* There are cases where the distinction made above between maternal effect and transmission is less applicable (e.g., inheritance of the transposable P element in *hybrid dysgenesis* in *Drosophila*, Engels, 1983).

Maternal Effects. A classic and dramatic example of maternal effect involves the direction of the shell spiral in *Limnea* (see Waddington, 1939). In this snail, spiral direction is controlled by a single chromosomal locus with a dominant dextral allele, *D*, and a recessive sinistral allele, *d;* the direction of the spiral is also determined by the maternal phenotype. Thus sinistrality is genetically recessive, but homozygous (*dd*) eggs laid by a dextral snail (*Dd*) are right spiraling. On the other hand, eggs laid by a sinistral parent will develop dextrality or sinistrality according to the Mendelian genotype of the offspring themselves. In this and in many other cases, *the maternal effect extends only a single generation: dd* eggs laid by phenotypically dextral (*dd*) snails develop into left-spiraling progeny.

The developmental behavior of this system may be rationalized as follows. In the absence of a positive regulatory factor derived from the *D* allele, a sinistral condition develops. A single dose of the *D* factor is sufficient to generate a dextral morphology; this dose need only be present during very early developmental stages.

Mta Is Not Dependent upon Simple Maternal Effects. The persistence of Mta phenotype through many generations of backcrossing is indicative of maternal transmission rather than maternal effect. In addition, Fischer-Lindahl and co-workers have performed several difficult and important experiments that bear on the question of maternal effects. Two possibilities are that the intrauterine environment conveys some information to the embryo or pup that modulates the expression of the Mta surface antigen. However, full-term mouse pups delivered by hysterectomy and fostered on mice of discordant Mta phenotype express the Mta phenotype of their natural mothers (Fischer-Lindahl *et al.*, 1980; Fischer-Lindahl and Hausmann, 1983). Fischer-Lindahl and Burki (1982) demonstrated through embryo transfer experiments that Mta phenotype was determined by the actual maternal phenotype, and not the surrogate phenotype. Similarly, the same authors reconstituted lethally irradiated F_1 mice with lymphocytes of the alternative Mta phenotype. The phenotype of recovered lymphocytes from the chimaeric products was strictly dependent on the strain of origin and stable for at least 4 months. These experiments rule out the possibility of a milk-borne factor or an intrauterine factor that regulates Mta expression in the embryo or pup.

These experiments, especially the reconstitution experiment, also rule out vertical transmission of Mta via a transmissible virus, such as mouse mammary tumor virus (MMTV; Bittner, 1936; Lyons and Moore, 1965) which would be a case of maternal transmission rather than effect. More recently, we have shown that *Mtf* can be transmitted from one cell by cell fusion *in vitro* to another and thus does not require developmental programming (see below). Furthermore, these results implicate mitochondria themselves as the residence of *Mtf*.

The Mta Phenotype of Hybrid Cell Lines Is Strictly Concordant with Mitochondrial DNA Type. Mta is expressed by several cultured cell lines (Table II), including the 8-azaguanine-resistant plasmacytoma line X63-Ag8.653 (Kearny *et al.*, 1979; Smith *et al.*, 1983; Smith and Rich, 1985). This enabled us to construct cell hybrids between this Mta^a cell line and Mta^b fresh splenocytes, and thus to manipulate experimentally the mitochondrial DNA type of a cell (Smith *et al.*, 1983; M. M. Huston *et al.*, 1985; see Fig. 4 and Table III). The Mta^a parental line contains Type V mtDNA, while the NZB parental cells contained Type I mtDNA, distinguishable using the restriction enzyme *Hin*fI (Fig. 3). X63-Ag8.653 was fused with fresh NZB splenocytes and hybrids selected in medium containing hypoxanthine, ami-

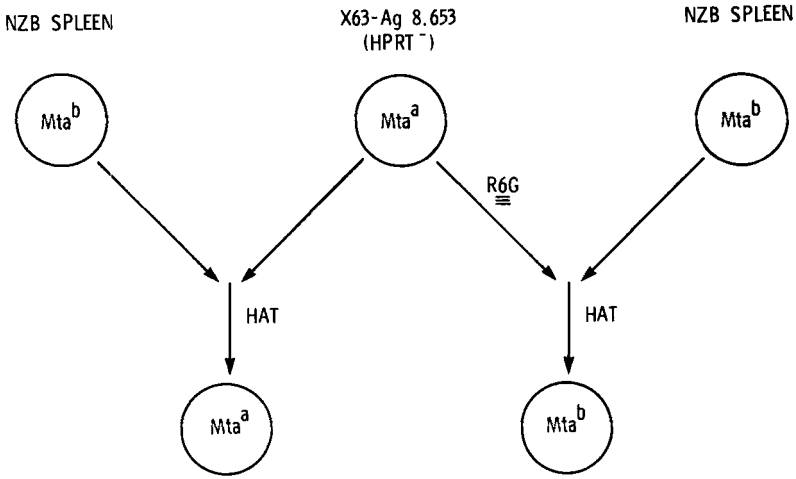

FIG. 4. "Maternal" transmission of Mta phenotype in somatic cell hybrids. The typical results of somatic cell hybridization involving fresh NZB splenocytes and the BALB/c plasmacytoma X63-Ag.653. In control fusions (left hand), most hybrids express Mta^a only, and contain Type V mtDNA only. When Type V mitochondria are poisoned with rhodamine 6G (R6G; right hand), most cell hybrids express Mta^b only, and contain Type I mtDNA only. In all hybrids, one parent acted as the maternal donor; no hybrids were detected that expressed both types of mtDNA or expressed both antigenic determinants.

nopterin, and thymidine (HAT). Hybrid cell lines were cloned by limiting dilution and shown to express nuclear antigens derived from both parental strains. Ten of these lines expressed Mta^a exclusively, one was shown to express Mta^b exclusively. (No cloned hybrids expressed both Mta phenotypes.) Restriction enzyme analysis of these cell lines indicated that Mta^a hybrid cells contained Type V mitochondrial DNA only, derived from the Mta^a parent cell. The single line expressing Mta^b contained Type I mtDNA only, derived from the Mta^b parent.

In these experiments, cell hybrids expressed one mtDNA (and one Mta) type or the other, but never both. This system thus represents a cellular analog of maternal inheritance of mitochondria and *Mtf* (see below). Under standard cell hybridization conditions, the X63 parent behaved in most cases as the "maternal" line, serving as the sole donor of both mtDNA DNA and *Mtf*. However, when the X63 parent was treated with 2 μM rhodamine 6G (R6G), a mitochondrial ATPase-specific poison (Gear, 1974; Ziegler and Davidson, 1981; Johnson *et al.*, 1980), the NZB parent usually acted as the "maternal" donor of both mitochondria and Mta phenotype (Table II). In the majority of these fusions (23/30=76%), poisoned X63 cells lost their maternal role in these experiments, while the NZB parent transmitted the Mta^b phenotype. In the absence of R6G, "maternal" transmission of *Mtf* through the X63 parent was 91% (10/11); in the presence of R6G, only 23% (7/30). This difference in transmission rate is highly significant ($\chi^2=14.3$; $df=3$; $p<0.001$). Type V mtDNA was transmitted in 80% of the hybrids sampled (4/5) in the control fusions and in 33% (1/3) of the R6G-treated hybrids. Thus it is clear that R6G modifies the transmission behavior of both *Mtf* and mtDNA in cell hybrids. More importantly, in every case a strict concordance was observed between the maternal transmission of mtDNA and maternal transmission of *Mtf*.

We do not know why the X63 parent was the predominant maternal donor of mtDNA and *Mtf* in the fusion experiments using untreated parents. There are really two distinct phenomena to be explained: why maternal transmission of either factor is observed; and why the observed transmissions are absolutely concordant? Interspecific cell hybrids between mouse and human almost invariably contain exclusively murine mitochondria (Clayton *et al.*, 1971; Attardi and Attardi, 1972) unless stochastic processes (DeFrancesco *et al.*, 1980; Giles *et al.*, 1980) or selective pressures (Wallace and Eisenstadt, 1979) lead to retention of the human chromosomes. Similar results have been obtained with rat × mouse hybrids (Hayashi *et al.*, 1980, 1982). Stable retention of both species of mitochondria was observed in mouse × hamster hybrid cells (DeFrancesco, 1983; Zuckerman *et al.*, 1984). A similar result was obtained when chloramphenicol-resistant (CAP^r) *M. domesticus* mtDNA was transmitted from enucleated cells (MTOC10, BALB/c origin)

TABLE II
Mta Typing of Cultured Cell Lines

Cell line	Cell type	Strain of Origin	Mta type	References[a]
ASL-1	Thymoma	A	a	(a)
AKR-A	Thymoma	AKR	a	(b)
BW5147	Thymoma	AKR	a	(a)
EL4	Thymoma	C57BL/6	a	(b)
HT-2	T cell	BALB/c	a	(a)
R1	Thymoma	C58/J	a	(c)
R1(T1-)	Thymoma	C58/J	Null	(c,d)
WEHI-105.7	Thymoma	NZB	b	(a)
YAC-1	Thymoma	A/Sn	a	(c,a)
6C10	CTL, Ly-2	(B10.Br × NZB)F_1	a	(a)
5F3	CTL, Ly-1,2	(NZB × B10.D2)F_1	b	(a)
F0	Myeloma	BALB/c	a	(c)
Pc10971[b]	Plasmacytoma	NZB/Icr	a-	(c)
Pc11198[b]	Plasmacytoma	NZB/Icr	a-	(c)
P3X	Plasmacytoma	BALB/c	a	(a)
P815	Plasmacytoma	DBA/2	a	(b)
X63-Ag.653	Plasmacytoma	BALB/c	a	(e)
WEHI 279	Plasmacytoma	NZC	a	(e,g)
MTOC10	Epithelioid mammary tumor	BALB/c	a	(a)
COMMA 1D	Mammary *epithelium*[c]	BALB/c	a	(f)
L (several derivatives)	Fibroblast	C3H/An	a	(a,b,f)
BE112	Fibroblast	NZB	b	(a,f)
BE(SV40)	SV40-transformed BE112	NZB	b	(f)
PCC4azaR1	Embryocarcinoma	129	Null	(f)
Y1	Adrenocortical tumor	(C57L/J × A/HeJ)F_1	Null	(f)

[a] References: (a) Smith *et al.* (1985); (b) Fischer-Lindahl *et al.* (1980); (c) Fischer-Lindahl and Langhorne (1981); (d) Fischer-Lindahl *et al.* (1983); (e) Smith *et al.* (1983); (f) J. R. Rodgers unpublished; (g) Ferris *et al.* (1983a).

[b] Reported typing used only Mta[a]-specific CTL.

[c] Danielson *et al.* (1984).

into *M. musculus molossinus:* even in the presence of chloramphenicol both species of mtDNA were maintained (Ho and Coon, 1979). In the reciprocal experiment the *M. domesticus* mtDNA was replaced entirely in the cybrid cells by *M. m. molossinus* mtDNA.

These results have been interpreted to indicate that maintenance of one species of mtDNA is dependent on species-specific nuclear gene products: when the responsible chromosomes are lost in cell hybrids that mtDNA type

TABLE III
MUTUALLY EXCLUSIVE TRANSMISSIONS OF BOTH Mtf AND mtDNA TYPE IN CELL HYBRIDS[a]

R6G treatment of Mta[a] parent	Mta type	Inferred Mtf	mtDNA type	(Number/total)
			Hybrids typed for mtDNA	
No	a	α	V	4/5
No	b	β	I	1/5
No	a + b			None
Yes	a	α	V	1/3
Yes	b	β	I	2/3
Yes	a + b			None
			All hybrids tested for Mta phenotype	
No	a	α		10/11
No	b	β		1/11
No	a + b			None
Yes	a	α		7/30
Yes	b	β		23/30
Yes	a + b			None

[a] Mta[a] X63-Ag8.653 plasmacytoma cells were fused with fresh NZB (Mta[b]) splenocytes and cell hybrids selected in hypoxanthine/amethopterin/thymidine (HAT) medium. In some cases the X63 parent was treated with 2 μ*M* rhodamine 6G (R6G) prior to fusion. Cloned cell hybrids were assayed for Mta phenotype using cloned CTL lines specific for Mta[a] or Mta[b]; selected hybrids were assayed for mtDNA type using silver staining of mtDNA treated with appropriate restriction enzymes. Data from Smith *et al.* (1983) and Huston *et al.* (1985).

cannot be maintained. The dynamics of mtDNA loss in interspecific hybrids may therefore depend on both the extent of trans-specific complementation of mitochondrial functions and the kinetics of chromosome loss. However, this explanation is unlikely to account for biased transmission observed in intraspecific hybridization experiments.

An alternative explanation is that the X63 tumor cells have more, or more actively replicating, mitochondria, and that NZB splenocyte mitochondria are lost due simply to stochastic processes. This last interpretation is strengthened by the single observation of maternal transmission of mtDNA from the NZB parent in the control fusions.

Both *Mtf* and mtDNA were transmitted in maternal fashion in these experiments, and transmission of each was affected in the same manner by R6G. In 8 cell hybrid lines in which both Mta phenotype and mtDNA type were determined, we observed concordant transmission in all 8 lines. The

sample size was too small to permit a statistical argument, but these results strongly suggest that *Mtf* and mtDNA are transmitted in a coupled fashion. However, we cannot exclude coincident but parallel behavior of these two factors.

The Mitochondrial Specificity of Rhodamine 6G Poisoning. In order to maintain the hypothesis of coincidental transmission bias, an independent sensitivity to R6G must be postulated for the putative *Mtf*. In fact, we know of no cellular function, other than the mitochondrial ATP translocase, that is inhibited by R6G. R6G is a positively charged lypophilic dye that binds preferentially to mitochondria (Johnson *et al.*, 1980) but exhibits some binding ability to rough endoplasmic reticular (RER) membranes (Terasaki *et al.*, 1984). The only known toxic effect of R6G is mediated by a potent inhibition of oxidative phosphorylation and mitochondrial ATP-dependent Ca^{2+} transport (K_i of 3 μM, Gear, 1974). The dye does not inhibit (uncoupled) submitochondrial ATPase or dinitrophenol-stimulated ATPase activities, indicating that membrane lipid interactions between the enzyme and the dye are required for inhibition. The positively charged analog rhodamine 123 (R123) also binds tightly to mitochondrial membranes, but is not toxic (Johnson *et al.*, 1980, 1981).

Treatment of CAP^r cell lines with R6G blocks the transfer of resistance (Ziegler and Davidson, 1979). CAP^r in mammalian mitochondria is linked to mtDNA restriction enzyme polymorphisms (Giles *et al.*, 1980; Ho and Coon, 1979; Wallace, 1981) and determined by point mutations in mtDNA (Blanc *et al.*, 1981; Kearsey and Craig, 1981). Since there is no known interaction of R6G with mtDNA itself, it is supposed that the mechanism by which CAP^r transmission is blocked is indirect. The most likely mechanism for the inhibition of mtDNA transmission may be that cells receiving a substantial number of poisoned mitochondria are impaired in their growth ability; in cells receiving only a few such mitochondria stochastic segregation processes eliminate the minor contaminants.

The confirmation that mtDNA itself contains *Mtf* will require the demonstration of genetic linkage of *Mtf* to a known mtDNA marker in cell fusion or cybridization studies. Alternate models are that extramitochondrial genetic elements interact in some restricted fashion with mtDNA, or that *Mtf* resides on intramitochondrial episomes (as does the cytoplasmic element believed to be responsible for cytoplasmic male sterility in maize, Laughnan and Gabay-Laughnan, 1983).

The Mta Phenotype of Mouse Strains Is Concordant with Mitochondrial Restriction Pattern Genotypes. Inbred and wild mouse populations differ with respect to mitochondrial DNA (mtDNA) restriction enzyme digestion patterns (Yonekawa *et al.*, 1980, 1981, 1982; Ferris *et al.*, 1982, 1983a, b, c). There are at least 31 such patterns within wild specimens of *M. domesticus*

alone, of which the standard form found in most "old strain" inbred mice (Type V, using the numerical designation of Ferris *et al.*, 1983a, see Table I) is relatively rare. Mitochondrial inheritance is strictly maternal in animals (*Drosophila,* Reilly and Thomas, 1980; *Xenopus,* Dawid and Blacker, 1972; rats, Kroon *et al.*, 1978; Hayashi *et al.*, 1978; Francisco *et al.*, 1979; mice, Avise *et al.*, 1979; equines, Yatscoff *et al.*, 1981; Hutchinson *et al.*, 1974; humans, Case and Wallace, 1981); this implies that each mitochondrial type represents a maternal lineage. More than a dozen separate maternal lineages have been found to express Mta^a; this includes lineages from other species of *Mus* (Ferris *et al.*, 1983a; see Table I).

Only Type I mitochondrial DNA has been associated with Mta^b (Ferris *et al.*, 1983a; Huston *et al.*, 1983). Type I mtDNA is found in all of the Mta^b strains listed in Table I; all of these are clearly or probably related through a maternal common ancestor within the last few decades. Wild mice bearing Type I DNA have been found sporadically in only two limited locations: a valley in California and farmland in Indiana (Ferris *et al.*, 1983c). Wild *M. domesticus* individuals bearing mtDNA restriction patterns very similar to Type I were trapped in Egypt. In addition, the subspecies *M. domesticus brevirostris* was shown to have a mitochondrial DNA pattern very similar to NZB (Yonekawa *et al.*, 1982). Unfortunately, none of these wild mice has been typed for Mta phenotype.

Recently, Fischer-Lindahl has discovered a third form of the antigen, Mta^c (Mtf^γ), in several strains of European wild mice (Fischer-Lindahl, 1983b; Table I). This explains at least some observations of partial killing (or partial inhibition in cold target blocking studies) using these strains (Fischer-Lindahl *et al.*, 1980). At least Mtf^α and Mtf^γ appear to be mutually cross-reactive on the basis of bulk CTL responses (Fischer-Lindahl, 1983a). The apparent cross-reactivities have not as yet been analyzed at the level of clonal CTL responses.

Just as Mta^b is restricted to a unique mtDNA restriction pattern, mtDNA from all the mice expressing Mta^c (Mtf^γ) exhibited a single pattern, even though the mice were obtained from locales widely separated geographically (Fischer-Lindahl, 1983a). The exclusive associations Mtf^β and Mtf^γ with rare mitochondrial DNA restriction patterns strongly suggests, but again does not prove, that there is a functional relationship between *Mtf* and mtDNA. We will discuss mechanistic models for a mitochondrial role in *Mtf* below (Section VI).

Ferris *et al.* (1983a) used methods of maximum parsimony tree analysis to generate lineage trees linking nine of the mtDNAs studied. These analyses suggested that Type I mtDNA was most closely related to Type II mtDNA found in the Mta^a strain SF/Cam. They inferred that a minimum of two mutations occurred during the evolution of Type I mtDNA from the putative

common ancestor of Type I and Type II mtDNAs. One of these, the *Fnud*II site at 9595 base pairs (Fig. 3; labeled FI) indicated a silent substitution in the unidentified reading frame 3 (URF3). The second site, the *Mbo*I site at 5016 base pairs (labeled MI), indicates a point mutation in $tRNA^{Ala}$. Neither of these changes suggests an obvious mechanism for *Mtf*. The sequence of a fragment from positions 4689 to 4900 revealed that the $tRNA^{Trp}$ gene in NZB mtDNA is identical to that in C3H mtDNA (Hirama *et al.*, 1983). Several point mutations were uncovered in URF2: two generated substitutions (Ile-Val; Ile-Thr); two were silent.

IV. Mta Shares Important Functional Properties with Class I Major Histocompatibility Antigens

A. Class I Genes and the MHC

A nuclear gene, *Hmt*, modulating the expression of Mta, has been mapped within the *Tla* region of the mouse MHC (Fig. 5). In addition, the immunological activity of Mta is similar to that of other class I antigens. Before reviewing these evidences, we will summarize the properties of class I and related genes. Recent reviews of this area have covered genetic, biochemical, and immunological aspects of class I antigens (Boyse, 1984; Hansen *et al.*, 1983; Hood *et al.*, 1983; Klein *et al.*, 1983; Weiss *et al.*, 1984).

Our understanding of the MHC has undergone two major developments during the last decade (Klein, 1982). Originally defined in terms of transplant rejection, the functions of both class I and class II *H-2* antigens were then recognized in terms of their abilities to present antigen to immune T cells during the course of normal immune function. Second, the discovery of additional alloantigens, the recognition of which was not *H-2* restricted, suggested that additional class I-like genes existed outside of the traditional boundaries of the major histocompatibility complex. These predictions were confirmed first by biochemical analyses, and more dramatically through molecular genetic techniques. Such analyses have led to the recognition that the genes encoding class I antigens constitute a large multigene family, only a few of which constitute the major transplantation antigens. A typical mouse chromosome 17 carries 25–30 class I genes, the majority of which map telomeric to the *H-2D* locus itself (Steinmetz *et al.*, 1982a; Winoto *et al.*, 1983; Mellor *et al.*, 1983; Weiss *et al.*, 1984; Rogers *et al.*, 1985—see Fig. 5). Class I genes known to be expressed include the classically defined *K*, *D*, and *L* genes, expressed on all somatic tissues, and *Qa-2*, *Qa-1*, and *Tla*, expressed on lymphoid tissues. Additional class I gene products have been detected in various tissues (Cosman *et al.*, 1982a, b; Kress *et al.*, 1983a, b; Lane *et al.*, 1984). The entire *H-2* complex is embedded in the *T/t* complex

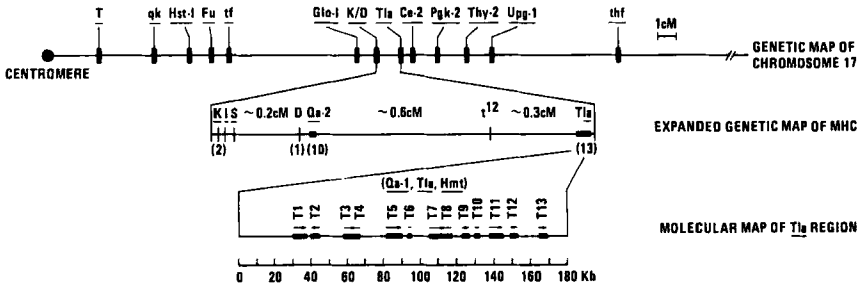

FIG. 5. Genetic and molecular map of the *Hmt* region of the MHC. The genetic regions surrounding *Hmt* are depicted at three orders of magnitude of scale. On the top map the centromer-proximal region of mouse chromosome 17 is labeled with genetic markers, and reflects a consensus. The central region from *K/D* to *Tla* is expanded to indicate the positions of the three clusters of class I genes and other markers of interest. The position of t^{12} is as found in the inverted *t* haplotypes (Shin *et al.*, 1984); this is likely to be the position in non-*t* chromatin. The numbers between loci indicate genetic distances in centimorgans (cM). *Hmt* has been mapped telomeric to *H-2D*, in the *Tla* region of the MHC. The *Tla* region of the C57BL/10 haplotype ($H\text{-}2^b$) is indicated in the lower map. This region has been mapped by overlapping cosmid clones; the distances shown are in kilobase pairs. At least three of the *Tla*-region genes are expressed in most strains: *Qa-1*, *Tla*, and *Hmt*. The relative positions of these genes is unknown. Maps are adapted from Lyon *et al.* (1979), Davisson and Roderick (1981), Steinmetz *et al.* (1982b), Weiss *et al.* (1984), Winoto *et al.* (1983), White *et al.* (1984), and Shin *et al.* (1984).

(Artzt, 1984; Shin *et al.*, 1984; Fox *et al.*, 1985). *T/t* mutants define altered cell surface antigens and disrupt embryonic development (Klein and Hammerberg, 1977). One mutation of interest with regard to the present subject is T^{hp}, which exhibits a dramatic maternal effect: heterozygotes are embryonic lethals if the mutant allele is inherited from the mother; they are completely viable if inherited from the father. This effect is probably not due to a cytoplasmic factor but to selective inactivation of paternal chromatin in the *T* region of chromosome 17 (Johnson, 1974, 1975; McGrath and Solter, 1984a, b).

B. Similarities between Mta and Class I Antigens

As yet, there are no direct biochemical properties known concerning Mta; such information will have to await the development of anti-Mta antibodies, acquisition of the relevant DNA sequence information, or development of novel biochemical techniques. Instead, the information we have is indirect and based on the behavior of Mta-specific CTLs.

1. Class I antigens are glycoproteins of molecular weight approximately 40,000–48,000. All of these are inserted into or secreted through the cell membrane via a cotranslational process involving recognition and subse-

quent trimming of a signal peptide (Walter *et al.*, 1984). The mature molecular weight is dependent somewhat on the presence of N-linked glycosidic residues.

We have no evidence concerning the molecular weight of Mta, but have investigated the possible involvement of glycosidic residues in Mta determinant expression. Jenkins *et al.* (1985) studied the sensitivity of Mta to tunicamycin treatment. This reagent inhibits the posttranslational addition of N-linked glycosides to membrane proteins (Struck and Lennarz, 1980). Tunicamycin treatment completely abrogates the ability of EL-4 thymoma cells to be lysed by certain Qa-1^b-specific cloned CTLs. In contrast, other cloned CTLs specific for Qa-1^b remain capable of lysing treated cells, indicating that tunicamycin treatment does not completely prevent the surface elaboration of the Qa-1 molecule itself. Similarly, treated cells remain completely sensitive to a Mta^a-specific cloned CTL (Fig. 6). These results suggest that the Mta.1 determinant is not dependent on N-linked glycosylation.

2. Class I antigens are noncovalently associated with β_2-microglobulin (Grey *et al.*, 1973; Vitetta *et al.*, 1975; Maloy *et al.*, 1984; for possible exceptions see Potter *et al.*, 1984; Rebai and Malissen, 1983). The third external domain of most class I antigens binds tightly with the 12,000 M_r β_2-microglobulin. Several lines of evidence indicate that Mta is also associated with β_2-microglobulin:

2a. Monoclonal anti-β_2-microglobulin blocks cell-mediated lympholysis specific for Mta (Fischer-Lindahl *et al.*, 1983). The monoclonal anti-β_2-m^b (anti-Ly-mll; Tomonari *et al.*, 1982) blocked anti-Mta^a CTL activity when assayed on C57BL/6 targets (H-2^b, β_2m^b) but not on C3H.SW targets (H-2^b, β_2m^a). However, a second anti-β_2-microglobulin monoclonal antibody (Chorney *et al.*, 1982) failed to block anti-Mta^a CTL activity. The authors suggested a difference in fine specificity of the two mAb could explain this discrepancy.

2b. Mta expression is expressed concordantly with β_2-microglobulin and H-2 class I antigens on different cell lines. Many cultured cell lines of diverse tissue origin express Mta (Table II). Three lines that do not express Mta are Rl-TL^- (Fischer-Lindahl and Langhorne, 1981; Fischer-Lindahl *et al.*, 1983), PCC4azaR1, and Y1 (JRR, unpublished observations).

Interestingly, all three of these lines are also unusual in their failure to express either K/D antigens or β_2-microglobulin. The β_2-microglobulin defect in Rl-TL^- (Hyman and Trowbridge, 1976; Hyman and Stallings, 1977) is apparently due to rearrangements in the structural gene on both chromosomes (Parnes and Seidman, 1982). The failure of PCC4azaR1 cells and other embryonal carcinoma (EC) cells to express class I antigens and β_2-microglobulin is well known and appears to reflect the developmental stage represented by these cells; instead they express the F9 or similar antigens (Artzt

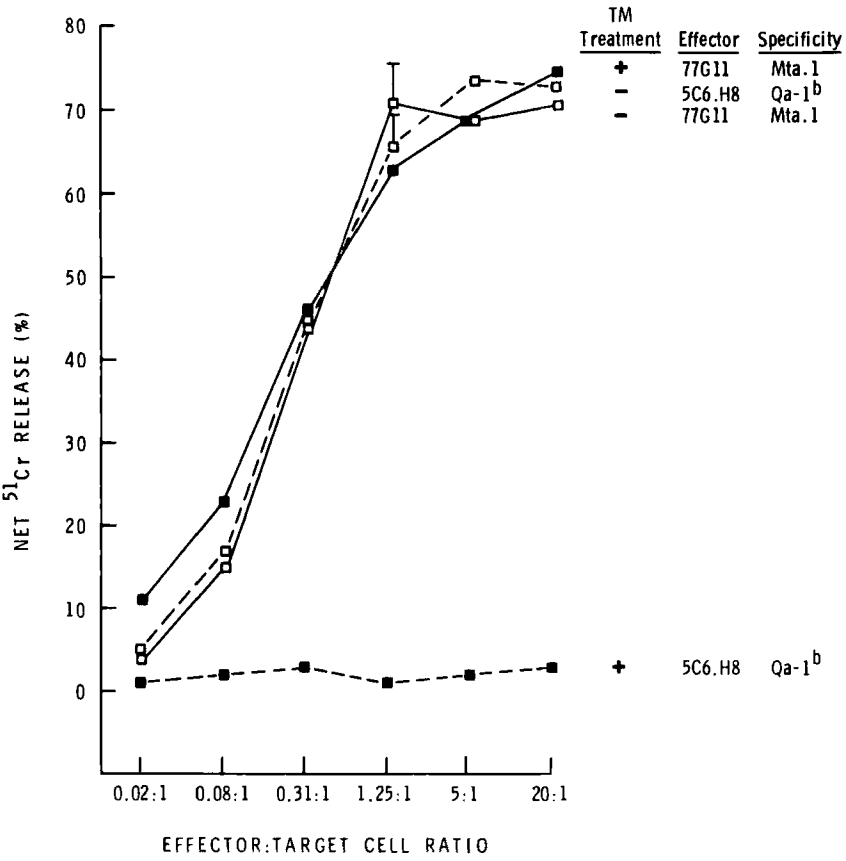

FIG. 6. Tunicamycin resistance of the Mta.1 determinant. EL-4 cells (Mta^a, Qa-1^b) were treated with tunicamycin and tested for susceptibility to CML by Qa-1^b-specific (5C6.H8; dashed lines) or Mta^a-specific (77G11, solid lines) CTL lines, in a standard 4 hour ^{51}Cr release assay. The Qa-1^b determinant detected by 5C6.H8 on control cells (open symbols) was completely obliterated in the presence of tunicamycin (solid symbols). In contrast, EL-4 cells were as susceptible to lysis by the Mta.1 CTLs in the presence as in the absence of tunicamycin (Jenkins *et al.*, 1985).

and Bennett, 1975). The defect in EC cells involves a failure to accumulate transcripts either of class I or of β_2-microglobulin genes (Croce *et al.*, 1981; Morello *et al.*, 1982; see Section V). The molecular mechanism involved in the failure of Y1 cells to express class I or β_2-microglobulin antigens is unknown. However, tumor cells readily give rise to derivatives lacking class I antigen expression after *in vivo* passage (Gooding, 1982; Rogers *et al.*, 1983; Seigler *et al.*, 1971; Gladstone and Pious, 1980; Pollack *et al.*, 1981) or *in vitro* immunoselection (Knowles and Swift, 1975; Hyman and Stallings,

1977; Gladstone and Pious, 1980). Regardless of the mechanism the concordant failure of these three lines to express class I MHC antigens and Mta strongly suggests a common mechanism in gene regulation and/or antigen biosynthesis.

3. All known class I antigen genes are located within the MHC on mouse chromosome 17. A survey of subspecies of *Mus musculus* revealed that two distinct *H-2* haplotypes found in V. Chapman's outbred colony of *Mus m. castaneus* at Roswell Park expressed no detectable Mta; the *H-2*-congeneic line B10.CAS2 (Zaleska-Rutczynska and Klein, 1977; Vucak *et al.*, 1984), which bears a third wild *H-2* haplotype, also is Mta^-. Mice with other *M. m. castaneus* *H-2* haplotypes did express Mta^a (Fischer-Lindahl and Hausmann, 1983; Fischer-Lindahl *et al.*, 1983). A Mendelian factor, termed *Hmt*, acted as a recessive allele in crosses involving the Mta^- and Mta^+ strains. *Hmt* segregated in F_2 progeny in a Mendelian fashion, and cosegregated with *H-2*-linked markers (Fischer-Lindahl *et al.*, 1983). Ten recombinants were obtained from almost 400 F_2 and backcross progeny, allowing assignment of *Hmt* to a locus 1.2 ± 0.6 cM telomeric to *H-2D* in the *Tla* region (Fischer-Lindahl and Robinson, 1983). Of these, one recombinant separated *Hmt* from *Qa-2*, placing *Hmt* telomeric to *Qa-2* (Fig. 5). None of the recombination events separated *Hmt* from *Qa-1* or *Tla*, while several recombinations separated *Hmt* from *Upg-1*. Therefore, *Hmt* is most likely located between *Qa-2* and *Upg*-1, and in the vicinity of the *Qa-1* and *Tla* genes.

Close linkage with other class I MHC loci is not, of course, evidence that *Hmt* is a class I gene. The *MHC* contains and is closely linked to other genes (Fig. 5; Klein *et al.*, 1983). Among others, these include several members of the complement cascade, steroid-specific cytochrome *P*-450 genes (White *et al.*, 1984), a group of liver-specific genes (Monaco and McDevitt, 1982), *tufted (tu)*, *Fused* (*Fu*, which exhibits a maternal effect, see McLaren, 1976, for review), and *Hst* (*Hybrid sterility*, Forejt and Ivanyi, 1975; Ivanyi, 1978; Forejt, 1981), genes affecting the rate of embryogenesis (Goldbard *et al.*, 1982), minor histocompatibility antigens, the enzymes glyoxalase (*G10-1*), cytosolic superoxide dismutase (SOD), and neuraminidase, a leukemia virus sequence (Meruelo *et al.*, 1984) and an α-globin pseudogene (Fox *et al.*, 1984). Another gene closely linked to *Hmt* that is of particular interest is t^{12} (and the probably identical t^{w32}) which maps between *Qa-2* and *Qa-1*/*Tla* in *t* chromatin, and probably in wild-type chromatin as well (Artzt, 1984; Shin *et al.*, 1984). This complex locus has been implicated in the expression of the F9 antigen expressed by early embryos, EC cells and male germ cells. Among the earliest pathologies in homozygotes are profound mitochondrial defects detectable as early as the two cell stage (reviewed by McLaren, 1976).

4. Class I antigens have a wide tissue distribution. K and D antigens are expressed on most somatic tissues from the 8 cell stage of development onward (Klein, 1982). In contrast, the *Qa/Tla* region antigens have a typically limited tissue distribution (Klein *et al.*, 1983). Qa-1, TL, and Qa-2 are essentially restricted to lymphoid tissue; Q10 to liver. Mta is found on both T and B lymphocytes (Fischer-Lindahl *et al.*, 1980; Smith and Rich, 1985) and on cultured cells from a variety of tissue types (Table II). The widespread expression of Mta on cultured nonlymphoid cell probably does not represent a feature of the transformed phenotype. The cell line Comma 1D, derived from normal mammary epithelia, exhibits highly differentiated hormone requirements and responses (Danielson *et al.*, 1984), and also expresses Mta^a (JRR, unpublished observations). Recently, we have obtained evidence that Mta is expressed by skin fibroblasts in primary culture (A. Han and J. R. Rodgers, unpublished observations). These results suggest that Mta, like *K/D/L* region antigens, is distributed on diverse somatic tissues.

5. Class I antigens stimulate primary responses *in vitro*. The generation of CTL reactivity to minor histocompatibility antigens requires primary immunization *in vivo* (Bevan, 1975). In contrast, alloreactivity against the K/D/L antigens can be elicited from primary MLC. However, most strains cannot consistently generate a primary response *in vitro* to allogeneic disparities within the *Tla* region (Widmer *et al.*, 1973; Bevan, 1975; Forman and Flaherty, 1978; Klein and Chiang, 1978; Kastner *et al.*, 1979a,b; Theofilopoulos *et al.*, 1979; Fischer-Lindahl and Hausmann, 1980). In contrast NZB mice are capable of generating a primary CTL acitivity specific for *Qa-1* disparities following *in vitro* sensitization (Botzenhardt *et al.*, 1978; Theofilopoulos *et al.*, 1979; Rich *et al.*, 1979; Fischer-Lindahl and Hausmann, 1980; Davidson *et al.*, 1981). For example, NZB cells ($H\text{-}2^d$, $Qa\text{-}1^a$, Mta^b), responding to BALB/c ($H\text{-}2^d$, $Qa\text{-}1^b$, Mta^a) stimulation *in vitro*, are able to lyse BALB/c targets, but BALB/c cells responding to NZB stimulators do not lyse NZB targets.

CTL recognizing Mta can be generated from primary mixed lymphocyte cultures (MLC; Smith *et al.*, 1982). Furthermore, splenocytes from (NZB × B10.D2)F_1 mice are capable of generating a potent CTL activity specific for Mta^a when sensitized *in vitro* with BALB/c splenocytes. Like the secondary responses, the primary responses are not restricted by the *H-2* haplotype of the target cells.

We find it heuristically useful to consider these differences in terms of a hierarchy; we do not necessarily impute mechanistic significance to this hierarchy (Table IV). In general, most strains can mount a primary response *in vitro* to allogeneic differences mapping to the *K/D* region, but not to *Qa/Tla* regional differences, including Mta. No strain combination has been found that generates a response to TL disparities, either *in vitro* or *in vivo*.

TABLE IV
HIERARCHY OF CLASS I CTL RESPONSES

Strain combination[a]	Antigenic disparity			Primary CTL response detected against		
	K/D	Qa-1	Mta	K/D	Qa-1	Mta
NZB anti-C57BL/10	+	+	+	+	–	–
NZB anti-B10.D2	–	+	+	–	+	–
(NZB × BALB/c)F_1 anti-B10.D2	–	–	+	–	–	+
(BALB/c × NZB)F_1 anti-B10.D2	–	–	–	–	–	–

[a] Primary responses against non-K/D disparities are not consistently observed from non-NZB strains. Maternal strain listed first in F_1 description.

NZB mice and some of their F_1 progeny can mount primary *in vitro* responses to Qa-1 and Mta disparities. Another feature of the hierarchical response to class I disparities is that the presence of allo-disparities at a higher level appears to inhibit the generation of simultaneous response to allo-disparities at a lower level. Thus, Qa-1 responses cannot be detected in the presence of K/D disparities. Similarly, Mta responses were not generated *in vitro* in the presence of Qa-1 disparities (Fischer-Lindahl *et al.*, 1980), but were obtained when the Qa-1 disparity was removed (Smith *et al.*, 1982). The response to Mta disparity is not completely dominated by Qa-1 disparity; secondary restimulation *in vivo* allows the generation of anti-Mta responses even in the presence of Qa-1 disparities (Fischer-Lindahl *et al.*, 1980).

6. Class I antigens provide targets for H-2 nonrestricted CTL activity. CTLs specific for a given class I antigen will lyse targets bearing that antigen, irrespective of the *H-2* haplotype of the target. In contrast, all other antigens (except the class II antigens) are recognized by specific CTLs only in the context of a specific class I antigen that was present on the stimulator cell (Klein, 1982).

Mta-specific CTLs are not H-2 restricted. CTLs generated against cells of a given *H-2* haplotype can detect Mta on cells from all other haplotypes. Thus, the only requirement for susceptibility to lysis by Mta^a-specific CTL is that a strain bear Mta^a. We have not listed the *H-2* haplotypes of the many strains listed in Table I, but it is apparent that these combinations include disparities at all the known polymorphic class I loci. *H-2* nonrestriction of Mta^b was confirmed by the use of F_1 backcrosses, which removed Type I mtDNA (NZB origin) from the *d* haplotype (Smith and Rich, 1985).

However, these observations cannot exclude the possibility that recognition of Mta is restricted by monomorphic, conserved regions of class I mole-

cules present in all class I antigens of all strains. The obligatory exception to the rule just elaborated actually provides a more compelling argument for non-*H-2* restriction. As mentioned, several wild *H-2* haplotypes found in *M. m. castaneus* are associated with resistance to Mta^a-specific CTL, even though these mice can be shown to carry *Mtf*$^\alpha$. These haplotypes express a full complement of *K/D* and *Qa/Tla* region antigens (Vucak *et al.*, 1984). However, one could argue that the hypothetical monomorphic restricting element present in all other strains is precisely absent in all the class I antigens of *Hmt*^{b} haplotypes. As already mentioned, recombinants have separated *Hmt*^{b} from the wild *H-2* region and placed it cis with respect to *H-2K*^{k} or *H-2D*^{k}. The latter *H-2* regions are associated with Mta expression in other recombinant inbreds (Table I) and thus should express the hypothetical monomorphic restricting domains. Nevertheless, animals with homozygous *Hmt*^{b} still fail to express Mta regardless of the *H-2* haplotype. This observation rules out the hypothesis that Mta is "restricted" to monomorphic determinants found on class I antigens of most haplotypes.

7. K/D region class I antigens determine the recognition of conventional antigens. In contrast, the antigens encoded in the *Qa/Tla* region have not been demonstrated to subserve this function, even in experiments specifically designed to demonstrate such functions (Kastner *et al.*, 1979b; D. L. Kastner and R. N. Jenkins, unpublished observations). These features are not unique to the mouse MHC; rat CTLs are predominantly restricted by the RT1.A and not by the RT1.C region class I antigens (Günther and Wurst, 1984). Although such experiments have not been conducted to detect a restriction function for Mta, it is unlikely that Mta normally subserves such functions. The evidence for this assertion is indirect and negative but nonetheless compelling. Most strains are monomorphic for Mta^a. Thus, if Mta^a did restrict conventional antigens, at least some CTL responses to conventional antigens would appear to be *H-2* nonrestricted. As already mentioned, all conventional antigens are *H-2* restricted.

8. At least several hundred alleles of K and D antigens occur naturally within the interbreeding species and subspecies of *Mus musculus* and *Mus domesticus* (Klein, 1982). The *Qa/Tla* region genes are unlike the *K/D* region genes in that very few alleles have been identified by serological and cellular assays (Flaherty and Lynes, 1984; Flaherty, 1984; Rich and Cook, 1984). In this respect Mta is more akin to Qa/TL genes than to K/D genes. Thus all of the strains and species of mice listed in Table I carry *Hmt*^{a}, while *Hmt*^{b} has been demonstrated in a few haplotypes of *M. m. castaneus*. (We cannot exclude, of course, polymorphisms that do not affect the expression of Mta.1 or Mta.2 determinants.)

Fischer-Lindahl has utilized the strain B10.CAS2 to screen for additional genetic variants at the *Hmt* locus. Variant stocks may be detected initially by

a partial or complete lack of expression of Mta. For example, *Mus spretus* splenocytes are less sensitive than BALB/c splenocytes to lysis by Mta^a-specific bulk CTLs, and are less efficient as inhibitors of lysis in cold-target inhibition assays (Fischer-Lindahl and Hausmann, 1983). Variant expression could be due to a nuclear gene defect (*Hmt*—or additional genes), or to a maternally transmitted factor (*Mtf*) defect. In order to distinguish between these possibilities, variant males were bred with B10.CAS2 females ($Hmt^{b/b}$, Mtf^{α}). If the variant male carried normal *Mtf* and a variant *Hmt* allele, Hmt^x the F_1 progeny would be ($Hmt^{x/b}$, Mtf^{α}). Variant expression of Mta in this F_1 generation would indicate a nuclear polymorphism. If the variant male carried a variant *Mtf* and Hmt^a, the F_1 generation would be ($Hmt^{a/b}$, Mtf^{α}) and would express Mta^a. Using this protocol an additional "silent" locus was found in wild *M. domesticus* males from Milan, and two expressing variant *Hmt* alleles were also identified (Fischer-Lindahl, 1983b; see Table I). *M. spretus* and *M. musculus bactrianus* carry a similar allele of *Hmt*, which has been termed Hmt^c (Fischer-Lindahl, 1983b).

V. Phylogenetic Considerations

The studies of mitochondrial DNA (mtDNA) restriction enzyme polymorphisms by Ferris *et al.* (1983a) suggest that Mta^b is present on only a few lineages of laboratory mice descended recently from a common mother. Of 31 mitochondrial DNA patterns determined by Ferris *et al.* (1983c), 9 have been assayed for the expression of Mta. Of these only one is associated with Mta^b; the remainder are associated with Mta^a (Table I). In addition, Mta^a is found in exemplars of numerous populations of *M. musculus* and *M. domesticus*. The closely allied form, Mta^c, was found in a few wild populations.

It is unknown whether Mta^b exists in the wild. Its expression in mice bearing a Robertsonian translocation of Sicilian origin [Rb(6.12)3Sic] may be due to NZB contamination during the establishment of the laboratory strain (Fischer-Lindahl and Hausmann, 1983). In a survey of 20 wild *M. domesticus* mice, Ferris *et al.* (1982) found 4 examples from two widely separated locales (California and Indiana) with mitochondria of the Type I pattern also found in Mta^b strains. Yonekawa *et al.* (1982) reported that *Mus domesticus brevirostris* has a mtDNA restriction pattern similar to that of the Type I NZB strain. This observation suggests that at least some *M. d. brevirostris* populations are Mta^b. In a survey of 208 animals from 8 *Mus* species, Ferris *et al.* (1983c) discovered an additional 5 animals from Egypt exhibiting the Type I mtDNA pattern. Egypt is also the source of 5 additional mtDNA patterns closely related to the NZB mtDNA pattern.

Other species of the genus *Mus* have been typed for expression of Mta.1 and Mta.2. Mta.1 has been detected in several subspecies within the *domes-*

ticus and *musculus* species, and in *M. hortulanus* and *M. spretus*. Detection of this determinant requires that the surface representation of the putative wild *Mtf* product in combination with the putative wild *Hmt* product produces a cross-reacting determinant. Detection of such cross-reactivity is, therefore, most easily interpreted as indicating closely related *Hmt* and *Mtf;* also possible are combinations of variant *Hmt* and *Mtf* that produce a cross-reacting combinatorial determinant.

Failure to detect cross-reactivity is less informative. The most obvious possibility is the absence of the appropriate determinants from the cell surface. Recent evidence suggests, however, that some CTLs may recognize class I allo-determinants only on cells of the species to which they were originally primed (Herman *et al.*, 1983; Bernebeau *et al.*, 1984a,b; Barbosa *et al.*, 1984; for a recent review of surface structures involved in T cell interactions see Goodman and Sercarz, 1983). The genus most closely related to *Mus* is *Rattus* (Brownell, 1983; Dickinson *et al.*, 1984). Splenocytes from several strains of rat were typed for expression of Mta^a with negative results, including Lewis, F344, Da, BN, Hooded Lister (Fischer-Lindahl and Hausmann, 1983). Harlan Sprague–Dawley was negative for both Mta^a and Mta^b (our unpublished observations).

VI. Models of the Genetic Mechanism of Mta

Non-Mendelian hereditary mechanisms pose special problems of genetic analysis. Experiments and observations concerning interspecific hybrids dating even before the rediscovery of Mendel's Laws revealed especially matroclinous (maternally transmitted) transmission of phenotype, providing in the early days as many counterexamples as examples of Mendelian inheritance. (For references to and discussions of earlier examples of maternal effects and transmission see Waddington, 1939; Wright, 1968.) McLaren (1962, 1979) has discussed criteria for evaluating possible cases of maternal effect and maternal transmission in mice. One of the earliest studied examples was mammary tumor incidence (Murray and Little, 1935); this of course is due to the milk-transmitted mouse mammary tumor virus, as shown by foster mothering (Bittner, 1936). Other cases of maternal effect or transmission in mice include the number of lumbar vertebrae in reciprocal crosses between C57 and C3H strains (Russell and Green, 1943; due to intrauterine environment, as shown by McLaren and Michie, 1958), the *Fused* locus, in which greater penetrance is observed in offspring of normal mothers than in offspring of *Fused* mothers (Reed, 1937; this gene maps within the T/t complex: Klein and Hammerberg, 1977), birth weight (Brumby, 1960), and number of vibrissae in the *Tabby* mutation (Kindred, 1961).

Plant and insect breeders have been familiar with cytoplasmic effects on

interspecific hybrid fertility for nearly a century (see Waddington, 1939; Wright, 1968). In numerous instances, reciprocal crosses differ dramatically in viability and fertility of the offspring. In some cases the effect is a purely maternal effect; in others incompatibilities between cytoplasmic genetic elements and nuclear genes have been strongly implicated. In the latter cases, cytoplasmic elements of one species are incompatible with nuclear genes of the other parental species. Matings using cytoincompatible females are inviable; the reciprocal cross is viable. In hermaphroditic plants the mitochondrial genes are implicated in numerous cases of *cytoplasmic male sterility* (*cms*, Edwardson, 1956, 1970). In maize, *cms* is associated with rearrangements of the mitochondrial genome and the formation of plasmid-like molecules (Laughnan and Gabay-Laughnan, 1983). The concordance of male sterility and mtDNA is intriguing, especially in light of observations of defective mitochondrial ATPases in *cms* (Barratt and Peterson, 1977). However, restriction enzyme site differences have also been observed in the chloroplast genomes of *cms* maize (Levings and Pring, 1979), raising the possibility that the organellar changes are only correlated with, and not the cause of, male sterility. This interpretation is supported by suggestions that both in barley (So, 1921; Imai, 1928) and in catnip (Woods and DuBuy, 1951) nuclear genes can induce chloroplast mutations; similarly, the maize nuclear gene *iojap* (Rhoades, 1950) can generate mutations in both chloroplast and mitochondrial genomes, including *cms* (Laughnan and Gabay-Laughnan, 1983).

Other systems of interest have generated scattered reports, and must be regarded as preliminary. These include evidence that self-perpetuating cytoplasmic factors can influence nuclear gene activation in somatic cell hybrids and cybrids (Gopalakrishan *et al.*, 1977; Gopalakrishnan and Anderson, 1979; Lipsich *et al.*, 1979; Giguere and Morais, 1981; also see John, 1984; Wilkie *et al.*, 1983).

Models of Mta must account for maternal inheritance, class I-like properties, and cotransmissibility of *Mtf* with mtDNA in cell hybrids. In addition, models invoking mitochondrial genetic elements must explain how genetic information within mitochondria is transmitted through at least the two mitochondrial membranes to be expressed on the cell surface. This is the topological problem. In the models presented below, we have indicated where precedents exist supporting the plausibility of the model; we are unaware of precedents for any models in their entirety.

We have been confronted with three types of models of Mta (Figs. 7–9). Models of the first class postulate that genetic control of Mta is entirely extramitochondrial. These models involve ad hoc explanations for the cotransmissibility of mtDNA and *Mtf*, but do not suffer from the topological difficulty. Models of the second type postulate mitochondrial residence for *Mtf*, but do not require that any macromolecular mitochondrial gene prod-

uct be exported. Instead, metabolic interactions between mitochondria and the nucleus induce the cell surface expression of a nuclear gene product. This model accounts for mitochondrial involvement of *Mtf*, and avoids topological problems. Such models can account easily for two alternate antigenic forms, but become cumbersome when three or more antigenic forms are involved. Finally, the third class of models postulates that *Mtf* encodes a structural or regulatory factor that traverses either two or three membranes. The *Mtf* gene product may interact with intracellular processes, or be represented on the cell surface directly. This model requires a mechanism for macromolecular export from mitochondria; at present such mechanisms are unprecedented, but offer no logical obstacles. We favor models which invoke mitochondrial genes; at this writing we are unaware of any evidence that conclusively excludes extramitochondrial models. We present alternative models not as strawmen, but because of the unprecedented nature of all the intramitochondrial models.

A. The Delbrück Model of Antigenic Variation (Class I Model; Fig. 7)

Delbrück (1948) offered the following model, based on analogy to bacteriophage lysogeny, as a formal alternative to the hypothesis of "plasmagenes" (Beale 1952), equivalent to mitochondrial genes, as an explanation of the phenomena of antigenic variation in *Paramecia*. A similar model has been proposed hypothetically in several other systems (J. Preer, personal communication; Heslop-Harrison, 1967, among others). The model is quite powerful and is easily generalized to other cases of antigenic variation.

Delbrück argued that "several systems at dynamic equilibrium are capable of additional different equilibria under identical conditions. They can pass from one equilibria state to another under the influence of transitory pertubations" [translation]. Applied to genetic systems, this observation means that a genetic system might be able to generate several different steady states of antigenic expression under identical external conditions, each of which could be extremely stable. A transient perturbation of the system, however, might be sufficient to convert it rapidly from one state to another, after which another period of stable expression of the new antigenic type would be observed. These were the phenomena to be described in *Paramecium*. In the present case we are confronted with extreme stability of the alternate states; no case of Mta switching has been described.

Applied to the present case, we might hypothesize that two pseudoallelic loci, *A* and *B*, are present in all individual genomes (labeled *Hmt-A* and *Hmt-B* in Fig. 7). The immediate product of each locus is a genetic regulator element. The A gene regulator element serves to repress the transcription of the B gene. The A regulator may optionally or occasionally stimulate its own

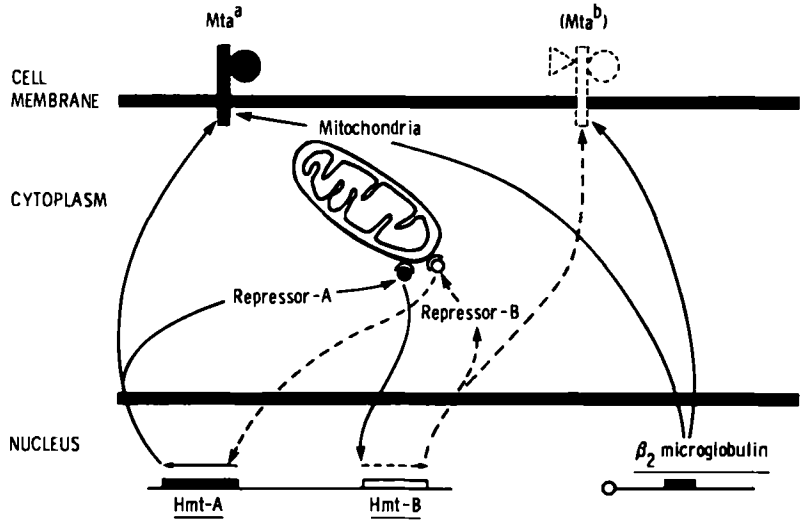

FIG. 7. Delbrükian model of Mta. An extramitochondrial model accounting for maternal inheritance of expression of Mta. The stable state expressing Mta^a is depicted. The products of a nuclear pseudoallelic locus, *Hmt*-A, include Repressor-A and a surface antigen. Repressor-A (solid lines) binds to a receptor on the surface of mitochondria, accounting for its maternal inheritance and for susceptibility to R6G poisoning. Repressor-A inhibits the expression of the alternative pseudoallele *Hmt-B*, Repressor-B, and the Mta^b surface antigen (dashed lines).

synthesis. The A gene regulator also stimulates production of a cell surface antigen, Mta^a. The B gene regulator represses A gene transcription and stimulates elaboration of Mta^b. This system can adopt one of two stable steady states: either A can be expressed with repression of B, or B can be expressed with repression of A. Additional loci can be accommodated easily by requiring that the A modulator inhibit all non-A genes, etc. Transitory environmental fluxes or stochastic processes may temporarily deplete the cell of both A and B regulators; at such a time both A and B will escape repression and be expressed. As soon as the regulators are synthesized they will begin to repress the alternate genes; eventually all but one gene will be repressed completely and a new stable state will be established. In the present case, the Mta regulators might be stable gene products present in the cytoplasm of all somatic cells and in ova. Sperm would contain little or no repressor material; hence embryos would always develop according to maternal phenotype. Such a mechanism would account for stable transmission of a given antigenic state over many generations.

To account for the behavior of Mta in cell fusion experiments, we hypothesize that the repressor factor would be bound to a mitochondrial surface receptor (Fig. 7). This feature has a teleological advantage in that the Mta

repressors would thus be assured representation in all viable daughter cells. Membrane disturbances by cationic lipophilic agents would then disrupt the activity of the Mta repressor.

Except for the *ad hoc* treatment of the R6G results, this model has certain precedents that suggest plausibility.

1. A genetic system sharing properties with the Delbrückian model may be found in the adenovirus EIA gene system. In particular, expression of EIA represses the expression of host class I genes, and in turn induces the expression of additional early functions, including the early gene E3, recently demonstrated to share primary sequence homology with genes of the immunoglobulin gene superfamily (Chatterjee and Maizel, 1984).

It has been suggested recently that the adenovirus early EIA gene product(s) are repressive regulators of transcription from genes whose transcription is enhanced by several different enhancer elements, including the EIA gene enhancer itself (Borelli *et al.*, 1984). Thus, regulation of EIA is autogenous; however, the EIA repressor is active in trans and the enhancer element can be separated from its gene without loss of function in either (Borelli *et al.*, 1984; Krippl *et al.*, 1984). The results of transfections of rat cells with the adenovirus 12 EIA gene indicated that the EIA gene product can also function as a repressor of class I histocompatibility genes (Schrier *et al.*, 1983); this effect was correlated with the high oncogenic potential of this gene (Bernards *et al.*, 1984). A similar correlation has been established in the case of the RadLV leukemia virus (Meruelo *et al.*, 1978, 1984).

According to the Delbrückian model illustrated in Fig. 7, the product of an *Hmt* pseudoallele not only would repress alternate pseudoalleles, but induce the expression of the Mta surface antigen. We have indicated in Fig. 7 the possibility that the surface antigen might also be a product of *Hmt* itself. It is also plausible that the regulatory factor which represses alternative pseudoalleles could stimulate expression from an additional locus containing the actual structural gene for the surface antigen. Again, precedent is set by the adenovirus EIA gene product(s). In addition to its repressive effect on genes using EIA, polyoma, and SV40 enhancers, the EIA gene product can stimulate other genes (Flint, 1982; Cross and Darnell, 1983; Leff *et al.*, 1984; Borelli *et al.*, 1984). Under certain conditions it may even stimulate expression of its own gene (Berk *et al.*, 1979; Nevins, 1981). The mechanism of gene activation by EIA gene product is unknown but may involve repression of the expression of other cellular repressors (Nevins, 1981).

2. Phase (antigen) variation of class I gene expression during early mammalian development may utilize similar repressive mechanisms. As already mentioned, EC cells do not express H-2 antigens (Artzt and Jacob, 1974; Forman and Vitetta, 1975; Dutko and Oldstone, 1981) or H-2 mRNA (Mor-

ello *et al.*, 1982). Cell fusion experiments suggest that the failure to express H-2 antigens is due to a dominant, trans-acting (diffusible) factor (Rousset *et al.*, 1979, 1980; Andrews and Goodfellow, 1980). The pattern of susceptibility to infection with murine cytomegalovirus (Dutko and Oldstone, 1981), Moloney murine leukaemia virus (Linney *et al.*, 1984), and polyoma virus mutants (Georges *et al.*, 1982; Vasseur *et al.*, 1982; Linney and Donerly, 1983) provided evidence that EC cells contain endogenous factors capable of repressing expression of viral genes similar to those inhibited by adenovirus gene EIA (Imperiale *et al.*, 1984). Differentiating agents that permit virus replication in EC cell derivatives also induce expression of class I H-2 antigens (e.g., Dutko and Oldstone, 1981). These observations suggest that class I H-2 genes may contain EIA-inhibitible enhancer elements (Borelli *et al.*, 1984), and that the same cellular factor that inhibits viral reproduction in EC cells is responsible for inhibition of class I *H-2* gene expression in these cells.

3. The maternal effects associated with hybrid dysgenesis in *Drosophila* is due to a maternally inherited repressor (Engels, 1983). Backcrosses in this system lead to a gradual diminution of dysgenesis. The distinction made earlier between maternal effect and maternal transmission cannot easily be applied in this system.

4. Mutually exclusive expression of a factor regulating egg–sperm compatibility has been proposed to account for maternal effects within the DDK system of hybrid inviability (Wakasugi, 1974). DDK mothers have low fecundity when mated to C57BL/6J (B6) males; the reciprocal cross is fully fecund. In addition, nonreciprocal segregation distortion is observed when F_1 mice are backcrossed to DDK or B6 mice. Wakasugi (1974) postulated that unlike other mouse strains, the DDK strain carries the *o* allele of the *om* (*o*vum *m*utant) locus which governs the synthesis of germ cell factors involved in egg–sperm compatability. +/+ eggs are compatible with all spermatazoa; *o*/*o* (DDK) eggs are incompatible with + spermatazoa. Wakasugi (1974) hypothesized that the paternal, but not the maternal, *om* locus is expressed in the zygote, accounting for the maternal effect on embryonic lethality. Wakasugi (1974) also proposed that the actual phenotype of an oocyte is determined not by the oocyte genome but by mutually exclusive deposition of *o* or + factor during oogenesis. (For additional examples of nuclearly encoded cytoplasmic factors affecting crosses between mouse strains see also Barton *et al.*, 1984; Muggleton-Harris *et al.*, 1982; Damjanov and Solter, 1982; Surani *et al.*, 1984).

Critique of the Delbrückian Model. The model of Mta regulation depicted in Fig. 7 cannot be excluded on the basis of available data and should be considered seriously. However, we have two principal objections to this model. Neither of these is compelling, but speak to the plausibility of the model.

The first objection to the Delbrückian model for maternal inheritance of Mta stems from consideration of the multistate problem. This model predicts for n different antigens, n different loci; for each locus, $n-1$ different repressor specificities. Thus the hypothetical regulator function of the *Hmt-A* gene should repress the enhancers of *Hmt-B* and *Hmt-C*, while the *Hmt-B* gene regulator should repress *Hmt-A* and *Hmt-C*. If additional antigenic forms of Mta are identified, this model will become more complicated.

The second objection is based on the supposition that transitory changes in the cellular environment could cause antigenic variation, much as transitory exposure to UV light induces expression of the lambda prophage in *E. coli*. Such a model suggests that individual cells might switch locus expression for stochastic reasons at a rate much higher than the mutation rate for individual genes, which is on the order of 10^{-6} per locus per cell generation (e.g., Puck, 1972). However, our preliminary (and unsuccessful) attempts to immunoselect for spontaneous or induced mutants suggest to us that the spontaneous rate of cellular conversion from one Mta state to another must be less than 10^{-7} (J. R. Rodgers, unpublished observations).

B. Mitochondrial Transmission with Induced Nuclear Response (Class II Model; Fig. 8)

Briefly, a mitochondrial gene defect at a functional locus (*Mtf*) produces a metabolic imbalance to which a regulatory system is responsive. In consequence, a compensatory nuclear gene, *Mta*, is activated. According to the model of induced antigen variation, the appearance of a surface antigen is linked to the expression of *Mta*. We favor the interpretation of an enzymatic or regulatory activity for the *Mta* gene product since both Mta^a and Mta^b are actual antigenic entities. The regulatory or enzymatic activities involved must be quite efficient, since surface expression of the Mta^a and Mta^b must be mutually exclusive in normal mice. If expression of *Mta* is dependent on mRNA synthesis and translation, metabolic inhibitors such as actinomycin D or cycloheximide might deplete the cell of the *Mta* gene product and allow dual expression and/or conversion of one form of Mta to the other. Our preliminary experiments with cycloheximide failed to demonstrate such conversion or dual expression (J. R. Rodgers, unpublished observations); however, the putative *Mta* product could be very stable. This model makes an interesting prediction concerning the behavior of cells engineered to contain both Mtf^{α} and Mtf^{β} (*Mtf*-heteroplasmic cells). Since the *Mta* activity is extremely efficient, it must be present in functional excess. Thus, either Mtf^{α} or Mtf^{β} should behave in dominant fashion in heteroplasmic cells.

Precedents for mitochondrially encoded metabolic disturbances are suggested by earlier reports that susceptibility to 6-aminonicotinamide-induced teratogenesis is maternally inherited in mice (Verrusio *et al.*, 1968; Gold-

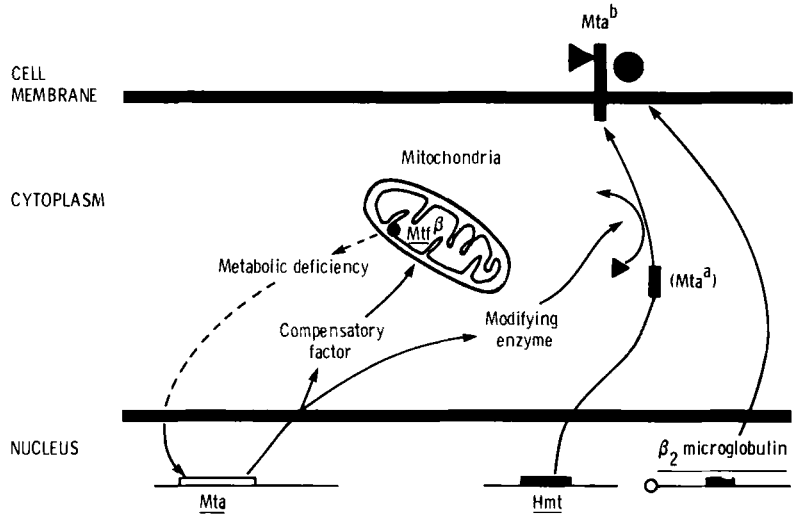

FIG. 8. Mitochondrial metabolic deficit with induced nuclear function. In the class II model depicted, NZB mitochondria are hypothesized to carry a defective gene, *Mtf*^{B}. The resulting metabolic disturbance (dashed arrow) induces a nuclear gene, *Mta*, which produces a compensatory factor. A by-product of *Mta* expression is the elaboration of a modifying enzyme, which converts efficiently the immediate product of the *Hmt* locus (Mta^{a}) to Mta^{b}.

stein *et al.*, 1963; for reviews see Biddle and Fraser, 1977; Morriss, 1979; these reports have not been confirmed). Nucleocytoplasmic interactions are plausible in this case because 6-aminonicotinamide is a potent inhibitor of mitochondrial oxidative phosphorylation (Coper and Neubert, 1964). Similarly, maternal inheritance of Leber's familial optical atrophy has been suggested on the basis of family studies (Cagianut *et al.*, 1981; Egger and Wilson, 1983). Patients with this disease have depressed levels of thiosulfate sulfur transferase (EC 2.8.1.1), a mitochondrial enzyme responsible for detoxifying cyanide, a potent inhibitor of mitochondrial respiration. The locations of structural or regulatory loci governing expression of thiosulfate sulfur transferase are unknown. A second human disease with possible maternal inheritance is the mitochondrial cytopathy syndrome, in which patients have myocardial deficits associated with structurally abnormal mitochondrial (Egger and Wilson, 1983). In contrast, plausible mitochondrial functions were not implicated in family studies suggesting maternal inheritance of retinoblastoma and the cancer family syndrome (Lynch and Guirgis, 1973; Kodama *et al.*, 1979) and in Huntington's chorea (Myers *et al.*, 1983).

This model accounts very easily for two features of the Mta phenomenology. The R6G data are explained simply by the assertion that *Mtf* is a mitochondrial gene. Second, the surface expression of Mta is explained by

asserting that it is a nuclear gene. The topological problem is avoided by the assumption that nuclear-mitochondrial communication involves indirect nuclear detection of metabolic deficiencies. The chief weakness with this model is that it does not account readily for the existence of additional alleles. For example, in order to account for *Mtf*^{v}, an additional metabolic disturbance is required with a compensatory nuclear response, once again triggering the expression of surface antigens.

C. Mitochondrial Transmission of an Enzymatic or Regulatory Activity (Class III Model)

In this model, a mitochondrial gene product is postulated that traverses both mitochondrial membranes and modifies or regulates a nuclear gene product. Again, the activity must be efficient, and dominant effects in heteroplasmic cells are predicted. In addition, the *Mtf* mRNA should be translated on chloramphenicol-sensitive mitochondrial ribosomes: chloramphenicol might block expression of Mta or convert one form of Mta to the other. Our preliminary experiments in this regard have indicated that exposure of splenocytes to chloramphenicol for 72 hours does not block or convert the expression of Mta (J. R. Rodgers, unpublished observations).

D. Mitochondrial Transmission of a Conventional Antigen (Class III Model; Fig. 9)

A simple version of this model postulates that *Mtf* is a mitochondrial gene encoding a polymorphic protein. This protein, or fragments of it, are exported through three cellular membranes to be represented externally in the context of the *Hmt* gene product. The simplest version of the model suggests that *Mtf* gene product fragments are exported through well-known mechanisms of intracellular protein degradation. Mitochondria decay metabolically; their proteinaceous contents must be digested (Holzer and Heinrich, 1982; Hare and Hodges, 1982). Some proteins are partially digested within mitochondria, perhaps by an inner membrane ATP-dependent endoprotease (Soler *et al.*, 1980; Desautels and Goldberg, 1982); fragments are probably degraded within lysosomes (Timoneda *et al.*, 1981; Soler *et al.*, 1980; Hernandez-Yago *et al.*, 1983). Experiments with extracellular conventional antigens have demonstrated that lysosomal processing is an obligatory step in antigen presentation; recycling of intralysosomal degradation products to the cell surface is implied (reviewed by Unanue, 1984).

Other versions of this model suggest that a mitochondrial deficit prevents biosynthesis of a substrate for class I antigen modification. We have evidence that Mta is not modified by N-linked glycosides (see above, Section IV), but cannot rule out other modifications.

The chief (empiric) difficulty with the conventional antigen model is the

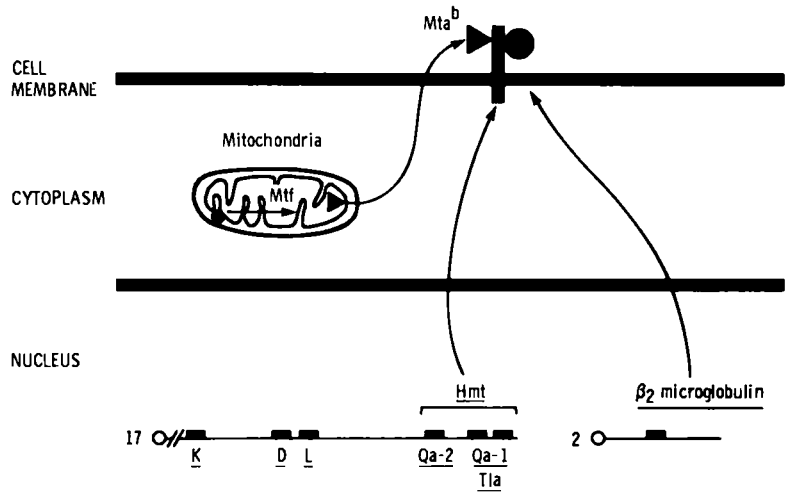

FIG. 9. Cell surface representation of a mitochondrial gene product. In this class III model, NZB mitochondria are hypothesized to synthesize a peptide or protein that traverses both mitochondrial membranes as well as the cell membrane. There it complexes with the *Hmt* product.

generalization that recognition of conventional antigens is restricted by *H-2* polymorphisms. We have reviewed the impressive evidence that Mta is not restricted by other MHC antigens (Section IV). Thus, this model implies that recognition of the *Mtf* gene product is restricted by the *Hmt* gene product, in the context of which recognition of other conventional antigens is unknown. Furthermore, the predominant response to Mta is not restricted by the K/D/L antigens, in the context of which all other conventional antigens are recognized. We cannot of course exclude the possibility that this relationship is entirely accidental; however the fact that three different forms of *Mtf* are each restricted in this unusual fashion strains complacent acceptance of this model. Thus, if *Mtf* is indeed a conventional antigen, it is likely that *Mtf* is not the accidental degradation product of a traditional mitochondrial gene product, such as cytochrome *B*. Rather, *Mtf* must encode a novel factor, the antigenic properties of which have been exquisitely selected by evolutionary processes to be restricted selectively by *Hmt*. In other words, the *Hmt* gene product would be a specific receptor for the *Mtf* gene product(s). The theoretical role of such a receptor has been discussed by John (1984).

It is intriguing, in this regard, that the *N*-formylmethionyl (NFM) peptide chemoattractant receptor (Snyderman and Pike, 1984) on neutrophils specifically and functionally binds purified mitochondrial gene products (Carp,

1982). This is not surprising, as mitochondria initiate protein synthesis with NFM residues, as do prokaryotes. If NFM peptides survive lysosomal processing, their expression on the cell surface might be deleterious. Perhaps *Hmt* is part of a scavenging system that specifically binds and neutralizes the *N*-formylmethionyl peptides derived from endogenous mitochondria. An alternative view is that *Hmt* restricts antigens encoded by intracellular prokaryotic parasites, just as other class I antigens restrict viral antigens. Intracellular prokaryotic parasites might not normally synthesize antigens reaching the host cell surface; fragments of such parasitic antigens could be processed via lysosomes and be expressed on the cell surface. There are many examples of parasitic, mutualistic, and symbiotic interactions between eukaryotes and intracellular prokaryotes (Margulis, 1970; Dodson, 1979); *Hmt* might be the product of a long evolution of cell surface structures capable of monitoring the condition of intracellular endosymbionts.

E. Mitochondrial Transmission of Class I-Like Antigen (Class III Model)

The problem of selective restriction of the *Mtf* product is sidestepped by this model: *Mtf* encodes a molecule which itself binds β_2-microglobulin and generates a class I-like cellular response unrestricted by *H-2;* this model ignores the role of *Hmt*. This model does require novel mechanisms for transporting such a molecule to the cell surface.

We have searched for homologies between class I antigens and all of the unidentified open reading frames (URFs) using computer programs developed by Dr. C. Lawrence. Using these programs we have discovered significant homology between a repetitive element found in the 3′ untranslated region of the cDNA clone pH^d-3 (Lalanne *et al.*, 1982; probably H-2-K^d, Kress *et al.*, 1983a) and mouse URF-6. The element is repeated several times throughout URF-6. At this time we do not attach functional significance to this finding. This was the only homology discovered at the level of nucleotide sequence; however we have not extended this search to the amino acid level. Since mitochondria utilize a slightly different genetic code it is possible that long evolution of a class I-like gene within mitochondria would select for considerable change in the nucleotide sequence and still maintain amino acid homology.

VII. Generation of CTLs Specific for the Maternally Transmitted Antigen

In this section we wish to review strain combinations which have proven useful in generating Mta-specific CTL, and to describe the effector cells involved in the response. Details concerning the generation of CTL lines

and clones specific for Mta are described in Jenkins and Rich (1983) and Smith and Rich (1985) (for review of CTLs and CTL cloning technology see Fathman and Fitch, 1982; Nabholz and MacDonald, 1983).

A. Strain Combinations

In Table I we have listed the laboratory strains that have been typed for Mta phenotype. Only a few strains express Mta^b, so immunization strategies must revolve around these lines. In general, there are two useful strategies for obtaining Mta-specific CTLs in bulk culture. The first makes use of two strains of NZB mice that carry different mitochondria and differ in Mta phenotype but are similar at most (but not all) nuclear loci (D. Lewis and N. Warner, personal communication). For example, Fischer-Lindahl has used the NZB/Bom (Mta^b) anti-NZB/Füll (Mta^a) combination to generate bulk CTLs specific for Mta^a (Fischer-Lindahl *et al.*, 1980). Such CTLs will lyse cells from all Mta^a strains regardless of their *H-2* haplotype. However, the reciprocal strain combination, NZB/Füll anti-NZB/Bom, is apparently ineffective in generating anti-Mta^b CTLs. In addition, these two strains do have minor background disparities that will generate CTL responses not specific for Mta (Fischer-Lindahl *et al.*, 1980); this problem might be obviated by cloning specific CTLs from the bulk cultures.

The second strategy makes use of the unique properties of maternally transmitted characters: reciprocal F_1 hybrids differ at cytoplasmically inherited loci but carry identical nuclear genes. Thus an efficient anti-Mta response is generated by F_1 antipaternal or F_1 antireciprocal F_1 reactions. The use of F_1 antipaternal reactions have the theoretical advantage that a larger T-receptor repertoire is available due to heterozygosity at T-variable region loci.

Fischer-Lindahl has used the outbred strain NMRI/Bom and the strains NZO/Dus, NZB/ Bom, and NZB/B1NJ, among others, for generating bulk culture CTLs. In particular, she has used the strain combinations NZB/Bom anti-NZB/Füll, and (NMRI/Bom×C57BL/6)F_1 anti-C57BL/6 to generate CTLs with specific cytotoxicity for Mta^a cells.

Our laboratory has used the combination (NZB×B10.D2)F_1 anti-BALB/c to generate primary responses *in vitro* (Smith *et al.*, 1982) and cloned CTL lines (Smith *et al.*, 1983). CTL clones have also been generated from NZB/B1NJ anti-BALB/c and NAB/B1NJ anti-B10.D2 secondary responses (Huston *et al.*, 1983). We have generated Mta^b-specific CTL clones using the reaction of (B10.BR × NZB/B1NJ)F_1 female anti-NAB/B1NJ male (Smith and Rich, 1985; Huston *et al.*, 1985).

The specificity and identity of anti-Mta CTL are demonstrated by differential lysis of reciprocal F_1 targets, and by lysis of cells from appropriate

strains or backcross mice (Smith and Rich, 1985) or, potentially, from mice of the recombinant inbred (RI) strains derived from C58 (H-2^k) and NZB (H-2^d) by Riblet *et al.* (1980; Fischer-Lindahl *et al.*, 1980).

B. Phenotype of Mta-Specific CTL

Mta-specific CTLs bear Thy-1 antigen as demonstrated by depletion analysis of bulk cultures (Fischer-Lindahl *et al.*, 1980) and flow cytometric analysis of cloned CTLs (Smith and Rich, 1985). Cloned CTLs are Ly-2^+; expression of Ly-1 varies between CTL clones (Smith and Rich, 1985). These characteristics are typical of the class I specific or restricted killer subclass of T lymphocytes (Rich and Rich, 1983; Burakoff *et al.*, 1984).

C. Natural Killer (NK) Activity of CTL Lines and Clones

In the course of typing cell hybrids using the X63-AG 8.653 plasmacytoma fusion partner we discovered that some, but not all, Mta^b-specific CTL lines would lyse certain inappropriate targets (Smith and Rich, 1985). In particular, these lines would lyse the NK target YAC-1 lymphoma line (Mta^a) and some of the Mta^{b-} cell hybrids. The appearance of NK-like activity in long-term CTL lines has been reported by other investigators (Acha-Orbea *et al.*, 1983; Brooks, 1983). We routinely include an Mta^a NK target (such as YAC-1) in our assays to control against NK-like activity by Mta^b-specific CTLs. As a control for similar activity in Mta^a-specific CTLs we have made use of the fact that embryocarcinoma cells are highly susceptible targets for NK lytic activity (Stern *et al.*, 1980). As discussed earlier, the EC cell line PCC4-azaR1, derived from strain 129, does not express either Mta^a or Mta^b. We have never detected NK-like activity in our Mta^a-specific lines.

VIII. Conclusions

A. Summary of Known Facts

1. Mta is an antigen on the cell surface. Mta is detected by cell mediated lysis assays (Fig. 1). Mta is detected on fresh and cultured T and B splenocytes and on cultured cell lines of diverse tissue origin (Table II). Only three cell lines of over a score analyzed fail to express Mta; these cell lines also fail to express β_2-microglobulin and the K and D class I antigens.

2. Mta is polymorphic within mouse strains (Table I). Two distinct forms have been well-characterized; at least one additional form has been identified recently (Fischer-Lindahl, 1983a). Mta^a is the form originally defined by secondary mixed lymphocyte responses in NZB/B1NJ anti-BALB/c reactions (Fischer-Lindahl *et al.*, 1980). Mta^a is also found on several subspecies

of *Mus domesticus* and in several other species, including *M. spretus, M. musculus castaneus, M. musculus molossinus,* and *M. hortulanus-pancevo.*

The form Mta^b has been detected only on strains and substrains closely related to NZB/B1NJ, and is associated exclusively with a unique restriction enzyme pattern of mtDNA. Similarly, Mta^c was found in two wild populations that share another unique mtDNA restriction enzyme pattern.

3. Mta is maternally transmitted. Reciprocal F_1 progeny with one NZB parent express and transmit only the maternal antigen. This transmission is stable for at least 11 generations of backcrosses and cannot be mimicked by embryo transfer or lymphocyte reconstitution of an irradiated host. This property defines a factor, *Mtf* (*m*aternally *t*ransmitted *f*actor).

4. Expression of *Mtf* is associated with mitochondria. The only known cytoplasmic genetic elements that share the maternal transmission properties of *Mtf* are mitochondria. Furthermore, cell hybrids generated using Mta-disparate parents expressed only the Mta determinant concordant with their mtDNA type. Treatment of one parent with the mitochondrial-specific poison rhodamine 6G (R6G) inhibited the transmission of both mtDNA and Mta phenotype in concordant fashion.

5. Expression of Mta is dependent on an *H-2*-linked locus, *Hmt*. This locus has at least three identified alleles. Hmt^a is the common allele found in most laboratory strains. Hmt^b is an apparently null allele found in three separate *H-2* haplotypes in *Mus musculus castaneus*. A variant allele, Hmt^c, is found in *M. spretus*.

6. The surface antigen recognized by Mta-specific CTL appears to be a class I MHC antigen. Recognition of Mta is not restricted by *H-2* haplotype, a property unique to class I (class II) MHC antigens. Furthermore, NZB mice are capable of mounting a primary response *in vitro* to Mta disparity, a property unique to class I MHC antigens. The tissue distibution of Mta is identical to that of class I antigens. Moreover, a monoclonal antibody recognizing β_2-microglobulin, which is tightly associated with class I (but not class II) MHC antigens, blocks CTL recognition of Mta. The association of Mta with β_2-microglobulin is confirmed by the expression of Mta on the thymoma R1, but not on the β_2m^--mutant line R1-TL$^-$. Similarly, Mta is not expressed on two other cell lines defective in β_2m and class I expression, PCC4 embryonal carcinoma cells and the Y1 tumor line.

B. Future Prospects

We have reviewed the phenomena of Mta and several mechanistic models that account for them. The evidence that Mta involves a class I antigen falls just short of being conclusive; compelling evidence would include a demonstration that the primary sequence of the surface molecule shares homology

with other class I MHC antigens. Due to the lack of antisera specific for Mta a molecular genetic approach is most likely to generate this evidence, probably using gene transfer studies involving recipient cells derived from the Hmt^b *H-2*-congeneic strain B10.CAS2 (Fischer-Lindahl and Steinmetz, 1983).

The role of mitochondria in Mta expression is strongly suggested by the mode of maternal transmission and the R6G sensitivity of transmission in cell fusion products. We cannot, however, formally exclude alternative models that do not involve intramitochondrial genes. Formal confirmation of mitochondrial involvement will require the demonstration of genetic linkage of *Mtf* to known mitochondrial DNA markers using somatic cell hybridization and cybridization techniques. Ultimately, such a demonstration will require the demonstration of recombination between marked mitochondrial genomes.

ACKNOWLEDGMENTS

Work from the authors' laboratory was supported in part by U.S. Public Health Service Grants AI 13810 and AI 18882. We thank Dr. R. Butow for helpful discussions of genetic models.

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ADVANCES IN IMMUNOLOGY, VOL. 38

Phagocytosis of Particulate Activators of the Alternative Complement Pathway: Effects of Fibronectin

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I. Introduction

Phagocytosis is an essential host defense mechanism that involves clearance of microorganisms and other foreign materials by blood and tissue leukocytes such as monocytes and macrophages. In mammalian species, monocytes are minor blood constituents which generally account for about 5% of the total circulating leukocytes and may be precursors of the more mature macrophages that accumulate in extravascular tissues (Volkman and Gowans, 1965; van Furth and Cohn, 1968). Adequate numbers of mononuclear phagocytes were first obtained from mice. Cohn and Benson (1965) developed methods for collecting and culturing macrophages from the peritoneal cavity of mice and made it possible to study phagocytosis at a cellular level. The subsequent development of isolation protocols for human pe-

ripheral blood monocytes (Böyum, 1968) has permitted studies with homogeneous human phagocytes within an existing framework of well-characterized responses obtained with mice.

Examination of the phagocytic capacities of human and rodent mononuclear phagocytes reveals fundamental similarities that underlie a unifying mechanism for cellular removal and disposal of foreign materials. The first and critical step is the capacity of phagocytic cells to distinguish foreign material from self components. This discriminating capacity is regulated by two distinct membrane receptors, the Fc receptor for IgG and the receptor for particulate activators of the alternative complement pathway. The Fc receptor (Berken and Benacerraf, 1966) is present on murine macrophages (Lay and Nussenzweig, 1968) and on human monocytes (Lobuglio *et al.*, 1967; Huber and Fudenberg, 1968) and recognizes the Fc portion of antigen-bound IgG; immunoglobulin molecules that lack the Fc fragment do not stimulate phagocytosis. The second receptor is the subject of this review. Unlike the Fc-IgG receptor, it directly stimulates phagocytosis of particulate activators in the absence of opsonins as initially noted by Rabinovitch and DeStefano (1973) for the ingestion of unopsonized zymosan particles by mouse macrophages. As will be discussed in Section II, the receptor for particulate activators recognizes the β-glucan constituent of zymosan and has recently been designated as the β-glucan receptor (Czop and Austen, 1985a). This receptor has been studied primarily with human monocytes (Czop *et al.*, 1978a,b) but is also present on human alveolar macrophages (Czop *et al.*, 1982b) and human neutrophils (Roos *et al.*, 1981; Williams *et al.*, 1985).

In addition to these two phagocytic receptors, murine macrophages (Lay and Nussenzweig, 1968; Beller *et al.*, 1982) and human monocytes (Huber *et al.*, 1968; Ross and Lambris, 1982) possess two distinct adherence receptors for cleavage fragments of the third complement component (C3), C3b and iC3b. Mouse (Blumenstock *et al.*, 1978b; Rourke *et al.*, 1984) and human (Bevilacqua *et al.*, 1981) mononuclear phagocytes also possess adherence receptors for fibronectin. The murine and human receptors for particle-bound C3 fragments are of major physiologic importance in augmenting phagocytic responses mediated by the Fc-IgG (Mantovani *et al.*, 1972; Ehlenberger and Nussenzweig, 1977) and β-glucan (Czop and Austen, 1980; Johnson *et al.*, 1984) receptors. The biological significance of the fibronectin receptor in phagocytic responses is less clear. Under physiologic conditions, native fibronectin does not bind to most particles; however, a specific cleavage fragment of human fibronectin selectively opsonizes particulate activators of the alternative pathway and augments their ingestion through the β-glucan receptor. These interactions are discussed in Section VII.

Once a signal has been received by a receptor that mediates phagocytosis,

the foreign material is surrounded by plasma membrane, enclosed, and internalized by the phagocyte within a vacuole, the phagosome. The processes involved with formation of the phagosome initiate activation of a variety of intracellular events which culminate in the fusion of the phagosome with membrane-enclosed granules that contain lysosomal enzymes (reviewed by Silverstein *et al.*, 1977). The fate of the ingested material within the newly formed phagolysosome depends on its susceptibility to the hydrolytic lysosomal enzymes and associated products. Ingestion also stimulates generation and release of other cellular metabolites. Intracellularly, these metabolites may facilitate destruction of the ingested material; extracellularly, they have the potential to stimulate and amplify inflammatory responses.

This review focuses primarily on nonimmune ligand–receptor interactions that mediate phagocytosis by mononuclear cells and secondarily on the cellular metabolites produced during the ingestive phase.

II. Phagocytosis of Unopsonized Zymosan by Human Monocytes

A. Composition of Zymosan

Zymosan is a yeast cell wall product of baker's yeast (*Saccharomyces cerevisiae*) initially prepared by Pillemer and Ecker (1941) and later named by SanClemente and Ecker (1943). As shown in Fig. 1, zymosan consists almost entirely of two types of carbohydrate polymers, β-D-glucans and α-D-mannans (Phaff, 1963; Bacon *et al.*, 1969). The β-glucosyl residues are consecutively joined by 1,3 and 1,6-glucosidic linkages with 1,3 branches (Misaki *et al.*, 1968), whereas the α-mannosyl residues are linked 1,6 and predominantly branched 1,2 (Lee and Ballou, 1965). Alkali treatment of cell walls of S. *cerevisiae* removes all of the α-mannans and yields particles composed solely of yeast β-glucans (Bacon *et al.*, 1969; Misaki *et al.*, 1968). These particles are similar in size to zymosan (3–4 μm in diameter) and are referred to as yeast glucan particles.

Zymosan particles have played a central role in the discovery (Pillemer *et al.*, 1954) and understanding of the molecular interactions of the serum proteins of the alternative complement pathway, an area recently reviewed by Kazatchkine and Nydegger (1982). The alternative pathway of complement affords the host a humoral mechanism of nonimmune recognition for foreign materials. Zymosan particles induce cleavage of C3 to soluble fragments, which are biologically active, and to particle-bound fragments, which can serve as ligands for C3 receptors. The practice of incubating zymosan with fresh serum produces opsonized zymosan which is coated with properdin (Pillemer *et al.*, 1954), C3 fragments (Goodkofsky and Lepow, 1972;

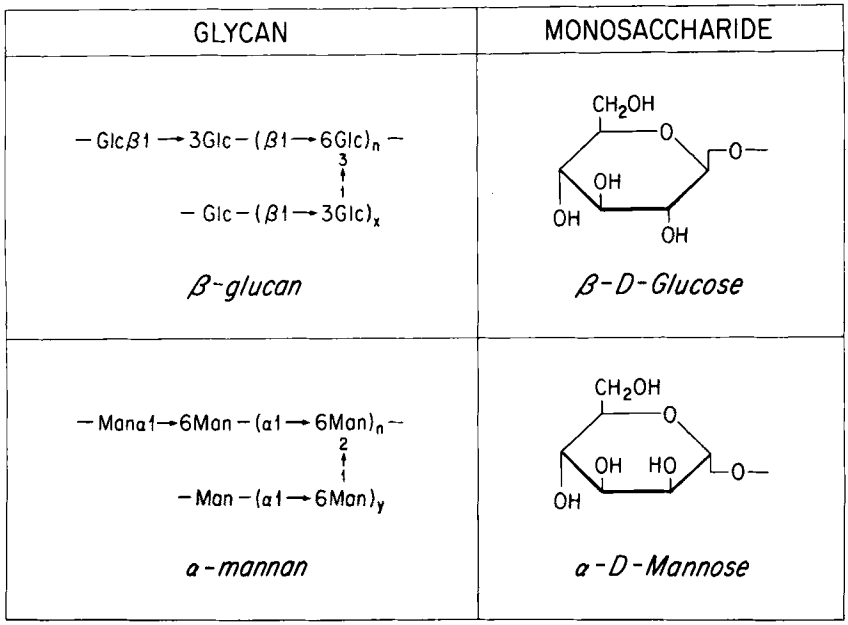


FIG. 1. Composition of the yeast cell wall product of *S. cerevisiae*, zymosan. Approximately 80–90% of yeast cell walls is carbohydrate that consists almost exclusively of the two polysaccharides depicted, namely, β-glucans and α-mannans. Unlike zymosan, which contains both of these glycans, yeast glucan particles contain only β-glucans.

Newman and Mikus, 1984), and serum immunoglobulins (Schenkein and Ruddy, 1981); these particles, therefore, display ligands for several different cell receptors. In contrast, unopsonized zymosan contains no alternative pathway or other serum proteins; with these particles, the carbohydrate polymers (glycans) and/or the structural conformations imposed by these chemical components are the only signals available to trigger cellular responses. Thus, under serum-free conditions, monocyte–zymosan responses represent relatively simple interactions and this review deals only with unopsonized zymosan particles.

B. Characterization of the Phagocytic Receptor

The capacity to ingest unopsonized zymosan particles is exhibited by all human cells that are morphologically and physiologically definable as monocytes. This is evidenced for monocytes separated by adherence from lymphocytes contained in mononuclear cell fractions (Czop *et al.*, 1978a) and for those purified by elutriation (Yasaka *et al.*, 1981) from granulocytes and lymphocytes (Czop, unpublished observations). The rate of ingestion is rapid and appears to be dependent *in vitro* on the time necessary to establish

contact between target and monocyte rather than on cellular secretory products or prolonged adherence. Ingestion by adherent monocytes of overlaid suspensions of zymosan particles is observed within 5 minutes of layering and reaches plateau levels involving nearly all of the cells within 15 minutes (Czop *et al.*, 1978a). Examination of suspensions of purified monocytes and zymosan particles, at target-to-phagocyte inputs of 10:1, reveals that essentially all of the zymosan particles are ingested within 1–2 minutes when contact is artificially induced by centrifugation at 40 *g*.

A prominent characteristic of the human peripheral blood monocyte and human alveolar macrophage (Czop *et al.*, 1982b) receptor that initiates ingestion of zymosan is its exquisite lability to trypsin. Pretreatment of monocytes with low levels of affinity-purified trypsin (<10 μg/ml) markedly reduces the subsequent proportion of monocytes ingesting zymosan particles but does not affect Fc or C3b receptor mediated functions (Czop *et al.*, 1978a). The capacity to ingest zymosan is, however, completely regenerated by trypsin-treated monocytes within 24–48 hours of culture in serum-free media, a result compatible with the restoration of a membrane constituent receptor.

The availability of commercial preparations of several β-glucans and α-mannans has made it possible to study the effects of these zymosan-like constituents on monocyte ingestion of particulate activators (Czop and Austen, 1985a). Pretreatment of adherent monocytes with 100 μg/ml of barley β-glucan reduces the subsequent capacity of washed monocytes to ingest 5 × 10^6/ml (74 μg/ml) zymosan particles by 75% within 20 minutes, whereas 1 mg/ml of yeast α-mannan has no effect after pretreatment for as long as 60 minutes. The β-glucan effect is not attributable to cell death and is even more pronounced when this glycan is presented before and during incubation with zymosan particles. Under these conditions, 20–25 μg/ml of barley β-glucan reduces the number of ingesting monocytes by 80–85% (Czop and Austen, 1985c). The effects with yeast α-mannan are markedly different. Mannan does diminish monocyte phagocytosis of zymosan particles without affecting cell viability, but it is at least 150 times less active on a weight basis than barley β-glucan. At inputs of less than 2 mg/ml, mannan exhibits no inhibitory capacity; 10 mg/ml of mannan effects 50–60% reductions in the numbers of monocytes ingesting zymosan. The inhibitory capacity provided by mannan is completely inactivated by enzymes that have substrate specificity for β-glucans but not by those with specificity for α-mannans or α-glucans. Pretreatments with α-mannosidase or α-glucosidase have no effects on the inhibitory moieties in either α-mannan or β-glucan, whereas pretreatments with β-glucosidase or β-glucanase (laminarinase) totally abolish the active components in both α-mannan and β-glucan. Analysis of the neutral sugar composition of mannan reveals 3% glucose, a portion of which

is likely to be a yeast β-glucan contaminant with inhibitory activity. That the β-glucanase, laminarinase, is at least 7-fold more effective in abrogating activity than β-glucosidase suggests that at least one carbohydrate ligand recognized by monocytes is a β-D-glucosyl oligo- or polysaccharide with 1,3 linkages (Czop and Austen, 1985a).

To establish specificity, a variety of other glycans have been tested (Czop and Austen, 1985a,c). Homopolysaccharides composed of glucose, galactose, or mannose have been used to examine monosaccharide specificity of the "β-glucan" effect, whereas glucopyranosyl polysaccharides with different anomeric configurations (α or β), glucosidic linkages, and degrees of branching have been used to examine structural requirements. The effects of these glycans and their structural compositions are summarized in Table I. β-Glucans with either of the two linkages prominent in zymosan, namely 1,3 and 1,6, all inhibit monocyte ingestion of particulate activators, whereas the 1,4-linked β-glucan (cellulose) does not. Neither α-glucans (dextran T-70 and nigeran) nor homopolysaccharides containing epimers of glucose (galactan and mannan) are inhibitory. It is noteworthy that each of these non-inhibitory glycans contains 1,3 or 1,6 linkages. As shown in Table I, the most efficient β-glucan is laminarin, a low-molecular-weight glucan that is completely soluble. Monocytes pretreated with less than 50 μg/ml of laminarin and then washed exhibit 50% reductions in their ingestion of zymosan parti-

TABLE I

CAPACITY OF VARIOUS GLYCANS TO INHIBIT MONOCYTE INGESTION OF PARTICULATE ACTIVATORS OF THE ALTERNATIVE COMPLEMENT PATHWAY

Glycan	Predominant chemical composition	Concentration for 50% inhibition of	
		Zymosan	E^r
Laminarin	β-D-Glucose (1,3; 1,6)	<50 μg/ml	<50 μg/ml
Barley β-glucan	β-D-Glucose (1,3 and 1,4)	<100 μg/ml	<100 μg/ml
Pachyman	β-D-Glucose (1,3)	>100 μg/ml	NT[a]
Pustulan	β-D-Glucose (1,6)	>500 μg/ml	NT
Cellulose	β-D-Glucose (1,4)	NI[b]	NI
Dextran T-70	α-D-Glucose (1,6)	NI	NI
Nigeran	α-D-Glucose (1,3 and 1,4)	NI	NI
Agarose	α-L/β-D-Galactan (1,3 and 1,4)	NI	NI
Mannan	α-D-Mannose (1,6; 1,2)	7.6 mg/ml[c]	>10 mg/ml[c]

[a] NT, not tested.

[b] NI, noninhibitory at ≥2 mg/ml.

[c] This concentration is for α-mannan not treated with β-glucosidase or β-glucanase; after treatment with either enzyme, mannan at 7 mg/ml was noninhibitory.

cles, whereas those pretreated and maintained in 20–25 μg/ml of laminarin exhibit reductions of 90–98%. Of the glycans listed in Table I, laminarin is chemically and structurally most similar to yeast β-glucan. The availability of yeast glucan particles has made it possible to study these mannan-free targets. The interaction of monocytes with glucan particles stimulates a marked phagocytic response comparable to that seen with equal numbers of zymosan particles (Czop and Austen, 1985b). The phagocytosis of yeast glucan particles is inhibitable with low concentrations of barley β-glucans but not by high concentrations of yeast α-mannans. Thus, the monocyte receptor for particulate activators recognizes both soluble and particulate forms of structurally similar β-glucans but not α-glucans, galactans, or mannans.

The β-glucan receptor is distinct from monocyte Fc receptors for IgG (Czop *et al.*, 1978a; Czop and Austen, 1985a). Particulate immune complexes containing sheep erythocytes (E^s) and 7 S anti-E^s are especially good indicator particles for identifying Fc-mediated responses. These E^sIgG can be conveniently prepared with highly purified IgG and are easily visualized within phagocytic cells. The IgG present on these indicator targets is the only potential ligand capable of eliciting a phagocytic response since E^s are not particulate activators of human (Platts-Mills and Ishizaka, 1974) or mouse (Polhill *et al.*, 1978; van Dijk *et al.*, 1980) alternative pathway complement proteins. The Fc-IgG receptor is present on all human monocytes (Ehlenberger and Nussenzeig, 1977; Czop *et al.*, 1978a), potentiates phagocytosis of E^sIgG at rates equivalent to those mediated by the β-glucan receptor for zymosan particles, and is functionally resistant to trypsin at concentrations 10-fold greater than that which inactivates the β-glucan receptor (Czop *et al.*, 1978a). Phagocytosis of E^sIgG is not affected by pretreatment of monocytes with α-glucans, α-mannan, galactans, or β-glucans (Czop and Austen, 1985a). The extent to which monocytes phagocytose E^sIgG provides a physiologic index of cell viability and this parameter becomes especially important in establishing that a test reagent inactivates receptors rather than cells. That β-glucans functionally inhibit monocyte ingestion of zymosan but not E^sIgG indicates preferential inactivation at the recognition level rather than at the ingestive phase. Aside from triggering phagocytic responses, the monocyte Fc and β-glucan receptors are similar in that they are both remarkably insensitive to manipulation during cell isolation and they both persist for at least 48 hours of culture in serum-free media (Czop *et al.*, 1978a; Czop and Austen, 1980).

The β-glucan receptor can initially be distinguished from monocyte receptors for C3b, now termed complement receptor type 1 (CR1), by the fact that the zymosan phagocytosed by monocytes neither carries nor displays epitopes equivalent to the ligand recognized by CR1. The ligand specificity of CR1 has been mapped to the C3c region of human C3b by the demonstra-

tion that $F(ab')_2$ fragments of polyclonal antibodies to C3c functionally block CR1-mediated adherence of C3b-bearing targets (Ross and Lambris, 1982). These $F(ab')_2$ fragments to C3c do not recognize zymosan particles and are routinely adsorbed with zymosan prior to CR1-blocking studies (Ross *et al.*, 1985). To distinguish β-glucan receptors from CR1 receptors, it becomes necessary to prepare indicator particles that carry only C3b. The most ideal particle is the sheep erythrocyte since, as noted above, it has an otherwise inert surface and since, C3b deposition on E^s is most comparable to opsonic deposition of C3b in physiologic situations. E^s bearing C3b are generally prepared by one of two methods. The first requires proteins of the classical complement pathway and the second proteins of the alternative complement pathway. By the first method, particulate immune complexes are prepared with E^s and 19 S anti-E^s (IgM) that has been rendered free of residual IgG. The E^sIgM are then sequentially incubated with functionally pure C1, C4, and C2 to form the classical pathway convertase, which cleaves added C3 to C3a and C3b. Much of the generated C3b binds to the particle which is then converted to E^sIgM,C4b,C3b by incubation in EDTA to remove C1 and C2. The second method for depositing C3b on E^s involves construction of particle-bound alternative pathway convertase, E^sC3b,Bb with purified human C3, B, and D. Incubation of this particle-bound C3-cleaving enzyme with additional C3 results in further generation and deposition of C3b. During subsequent washings, the Bb fragment spontaneously dissociates and the end product is E^sC3b. The preparation of E^sIgM,C4b,C3b requires several functionally pure proteins and relatively large amounts of purified human C3, whereas preparation of E^sC3b requires very large quantities of homogeneously pure alternative pathway proteins.

Both of these C3b-bearing particles are functionally identical and interact only with CR1. The CR1 receptor is present on all human monocytes (Bianco and Nussenzweig, 1977; Czop *et al.*, 1978b), promotes adherence but not phagocytosis (Ehlenberger and Nussenzweig, 1977; Czop *et al.*, 1978a), and is functionally resistant to trypsin at 10-fold higher concentrations than that which inactivates the β-glucan receptor (Czop *et al.*, 1978b; Czop and Austen, 1980). CR1-mediated functions are fully expressed by monocytes pretreated with β-glucans, α-glucans, α-mannans, or galactans (Czop and Austen, 1985a). The presence of C3b on IgG-coated targets (Ehlenberger and Nussenzweig, 1977) or on particulate activators (Czop *et al.*, 1978b; Johnson *et al.*, 1984) increases ingestion by improving contact between monocyte and target; however, synergy is not involved since in the absence of an independent phagocytic signal, C3b fails to initiate any ingestion. When either the CR1 or the β-glucan receptor is independently occupied by its respective ligand, there is no effect on the functional capacity of the other. This is illustrated by the following experiment. Monocytes rosetted with E^sC3b

exhibit no impaired capacity to ingest zymosan particles and the ingestion of zymosan by a monocyte with E^{s}C3b attached to its exterior surface via CR1 does not trigger phagocytosis of the C3b-coated erythrocytes (Czop and Austen, 1980). That CR1 and β-glucan receptors are independent entities is also evident with cultured monocytes. Unlike the phagocytic receptors for Fc-IgG or particulate activators, functional CR1 is completely lost within 48 hours of culture in serum-free media and this process is not accelerated by trypsin.

The β-glucan receptor is also distinct from the monocyte complement receptor type 3 (CR3); however, unlike CR1, CR3 regulates cellular adherence. This makes it more difficult to separate adherence functions mediated by CR3 from phagocytic receptors per se since phagocytosis is a contact dependent process. The ligand for CR3 is iC3b, which contains neoantigens generated during cleavage of C3b by C3b inactivator (Carlo *et al.*, 1979). The CR3 receptor does not recognize C3b, is not functionally inhibitable by antibodies to CR1, and is present on all human monocytes and neutrophils (Ross and Lambris, 1982). When iC3b is fixed to a target that otherwise lacks a phagocytic signal, such as E^s, the E^s-iC3b adhere with monocytes but are not ingested; the presence of iC3b on particulate activators augments their ingestion through the β-glucan receptor (Johnson *et al.*, 1984). Several monoclonal antibodies have been described that block iC3b-mediated rosetting. Among these are rat monoclonal M1/70 (anti-Mac-1) (Beller *et al.*, 1982), mouse anti-Mo1 (Arnaout *et al.*, 1983), and mouse anti-OKM1 and anti-OKM10 (Wright *et al.*, 1983). The rat monoclonal M1/70 has specificity for mouse peritoneal macrophage cell surface antigen (Mac-1) (Springer *et al.*, 1979), cross-reacts with human monocytes, neutrophils, and null cells (Ault and Springer, 1981), and immunoprecipitates two different-sized glycoproteins from macrophage membranes (Kürzinger and Springer, 1982). The larger of the two subunits is termed the αM polypeptide, has a molecular weight (M_r) of 155,000–170,000, and is noncovalently linked to the smaller β-polypeptide subunit of M_r 95,000 (Kürzinger and Springer, 1982; Sanchez-Madrid *et al.*, 1983). The mouse monoclonal anti-Mo1 was raised against human monocytes (Todd *et al.*, 1981) and recognizes human monocytes, neutrophils, null cells, human peritoneal macrophages, and monocytes treated with 1 mg/ml of trypsin (Todd *et al.*, 1981; Todd and Schlossman, 1982). It immunoprecipitates two glycoproteins from human monocytes that are comparable in their subunit composition and structure to those obtained from mouse macrophages with anti-Mac-1 (Todd *et al.*, 1982). It is noteworthy that anti-Mac-1 recognizes but does not precipitate the human CR3 receptor. Mouse anti-OKM1 (Breard *et al.*, 1980) and anti-OKM10 (Talle *et al.*, 1983) recognize the same cell types as anti-Mac-1 and anti-Mo1. The OKM1 epitope is apparently equivalent to Mac-1 and Mo1, whereas

TABLE II
EFFECTS OF ANTIBODIES TO COMPLIMENT RECEPTOR TYPE 3 (CR3) ON MONOCYTE PHAGOCYTOSIS[a]

Monoclonal antibody	Monocytes phagocytosing (%)			
	Zymosan particles		E^sIgG	
	≥1	≥3	≥1	≥3
None	67	25	79	58
Mac-1	67	21	9	2
OKM1	62	28	82	38
OKM10	71	26	83	55
AHF5[b]	60	20	72	37

[a] Preformed monolayers of human monocytes were treated at 25°C for 20 minutes with 8 μg/ml of each purified monoclonal antibody, decanted, incubated at 37°C for 30 min with zymosan or E^sIgG in 8 μg/ml of the same monoclonal, and assessed for the percentage of monocytes ingesting ≥1 and ≥3 targets. The zymosan particle to monocyte ratio was 5:1, whereas the E^s IgG to monocyte ratio was 25:1.

[b] AHF5 was used as a control. It is a rat monoclonal of the same class as Mac-1 (IgG_{2b}) that recognizes mouse kappa chains.

OKM10 is a different epitope on the OKM1/Mo1/Mac-1 molecule. Thus, each of these four monoclonal antibodies recognizes the αM-polypeptide subunit of CR3. A mouse monoclonal raised against human T-lymphocytes, TS1/18 (Sanchez-Madrid *et al.*, 1982), recognizes the β-subunit.

Monoclonal antibodies to CR3 receptors have been used to determine their effects on phagocytic processes. Although the reported capacities of these monoclonals appear to vary, examination of the experimental designs of these studies reveals a consistent pattern, namely CR3 receptors are important in cellular processes that are intrinsically contact dependent. One factor which contributes to the observed variability is the use of suspended or adherent phagocytes. When adherent monocytes in preformed monolayers are pretreated with saturating concentrations of purified intact monoclonal antibodies to CR3, the functional effects on Fc and β-glucan receptors are equivalent (Table II). There are no effects by any of the three anti-CR3 antibodies on zymosan ingestion and there are no specific effects on the phagocytosis of E^sIgG. The decreases shown in Table II with E^sIgG are indicative of the dual recognition by IgG monoclonals for αM epitopes and Fc receptors (Beller *et al.*, 1982). The use of suspended cells is much more likely to reveal inhibitory effects by these monoclonals since under these conditions many more contact-related events are required for phagocytosis.

Another factor which contributes to the observed variability in the capacities of monoclonals to CR3 to block phagocytic responses is the number of

functional ligands on the indicator particles. When E^s are sensitized with low numbers of IgG molecules and used as indicator targets, monocytes pretreated with $F(ab')_2$ fragments of anti-Mo1 exhibit a complete loss in Fc receptor-mediated phagocytosis. As the numbers of anti-E^s are increased, monocyte ingestion of E^sIgG becomes increasingly less inhibitable by these $F(ab')_2$ fragments (Dana *et al.*, 1984). A similar ligand-dependent blocking capacity is observed for phagocytic responses mediated by the monocyte β-glucan receptor (Ezekowitz *et al.*, 1983). In the absence of monoclonals to CR3, zymosan which has been rendered ligand deficient by oxidation and reduction is poorly ingested by adherent monocytes. After pretreatment with anti-Mo1, monocytes exhibit an even lower phagocytic response with these zymosan particles. Ligand-dependent phagocytic responses via Fc and β-glucan receptors are also observed for patients with CR3 deficits (Anderson *et al.*, 1984). Phagocytes from these patients exhibit reduced phagocytic, chemotactic, and substratum-adherence responses suggesting that CR3 receptors functionally contribute to each of these adherence-dependent processes.

Since the capacity of particulate activators to initiate a monocyte phagocytic response directly parallels their capacity to activate the plasma proteins of the alternative complement pathway (Czop *et al.*, 1978a), the question arises as to whether the β-glucan receptor is a membrane-associated alternative pathway protein. It is appropriate at this point to define the proteins in question and to provide some insights as to what is meant by the term particulate activator. The alternative complement pathway consists of six plasma proteins: P (properdin), C3b, B, D, H (β1H), and I (C3b inactivator). Four of these proteins, P, C3b, B, and D, are involved with formation of a C3-cleaving enzyme (convertase), whereas the remaining two, H and I, regulate convertase inactivation. By specific assays, it has been shown that C3b functions as a receptor for P (Goodkofsky and Lepow, 1972; Fearon and Austen, 1975; Schreiber *et al.*, 1975), B (Fearon *et al.*, 1973), H (Whaley and Ruddy, 1976), and I (Pangburn and Müller-Eberhard, 1978). P is not required for convertase activity (Fearon *et al.*, 1973) but C3b and B are essential. In the presence of magnesium ions, C3b and B form a complex, C3bB, which has partial convertase activity (Daha *et al.*, 1976a; Amos *et al.*, 1976). When complexed to C3b, B is cleaved by the serine protease D (Fearon *et al.*, 1974) to a small fragment (Ba) which is released and a large fragment (Bb) which remains bound to C3b (Müller-Eberhard and Götze, 1972; Fearon *et al.*, 1974). This C3 convertase, C3b,Bb, amplifies cleavage of C3 but, rapidly and irreversibly dissociates; it can be regenerated by the addition of B and D (Fearon *et al.*, 1973). Binding of P to the C3b in the C3bBb complex greatly reduces its rate of spontaneous dissociation (Fearon and Austen, 1975), thereby increasing the amount of C3 cleaved. In the

C3bBb complex, C3b is protected from inactivation (Daha *et al.*, 1976b); however, following its dissociation from this complex, C3b can either bind to B and generate another C3b,Bb complex or to H to form C3bH (Kazatchkine *et al.*, 1979). When complexed to H, C3b is rapidly cleaved by the serine protease I to iC3b, which does not interact with B (Harrison and Lachmann, 1980). Thus, H and I prevent C3 cleavage by inactivating one of the components essential for convertase formation. In the presence of particles, any generated C3b can bind to essentially any surface. The addition of B to particles bearing C3b generates a C3bB complex, addition of H generates a C3bH complex, and the addition of both B and H generates competition for the bound C3b (Kazatchkine *et al.*, 1979). Preference as to which will bind is determined by surface properties of the particles (Fearon and Austen, 1977). If a C3b,Bb complex is favored, the particle is said to be an activator since large amounts of C3 can be cleaved. If, however, the C3bH complex is favored, the particle is a nonactivator. The definition, therefore, of a particulate activator is that it is a particulate surface that induces cleavage of C3 by promoting formation and protection of particle-bound amplification convertase, C3b,Bb.

Two different types of studies have been undertaken to determine if the β-glucan receptor is a membrane complement protein. The first studies involve antibodies to complement proteins and the second, cations. In the first studies, adherent human monocytes were treated with each of five purified IgG antibodies that recognize human P, C3, B, H, or I. At inputs of as much as 4 mg/ml, none of these antibodies interferes with monocyte ingestion of zymosan particles. At target-to-monocyte ratios of 5:1, zymosan ingestion involved 70% of the monocytes and this proportion was reduced to no less than 60% regardless of whether monocytes were pretreated or pretreated and maintained in the antibodies during incubations with zymosan particles. Thus, although the β-glucan receptor is sufficiently exposed, as evidenced by its ready inactivation by trypsin, it does not interact with antibodies against P, C3, B, H, or I. These results, however, do not distinguish between the possibilities that membrane-associated complement proteins are not recognizable by immunologic probes raised against plasma-derived counterparts or are not involved in monocyte ingestion of particulate activators. Therefore, a second approach has been taken which obviates the use of antibodies. It had been demonstrated that C3b and B form a magnesium-dependent complex (Müller-Eberhard and Götze, 1972) and that the magnesium requirement can be substituted by other cations such as cobalt, but not by calcium (Wardlaw *et al.*, 1958; Lew *et al.*, 1975). Thus, to determine if a membrane-associated C3bB complex is the β-glucan receptor, adherent monocytes were incubated with zymosan in 5 m*M* veronal-buffered saline containing 0.1% gelatin (GVB^{2-}), pH 7.4, alone or with incremental increases of Mg^{2+}, Co^{2+}, or Ca^{2+}. $E^{s}IgG$ were used as controls. As

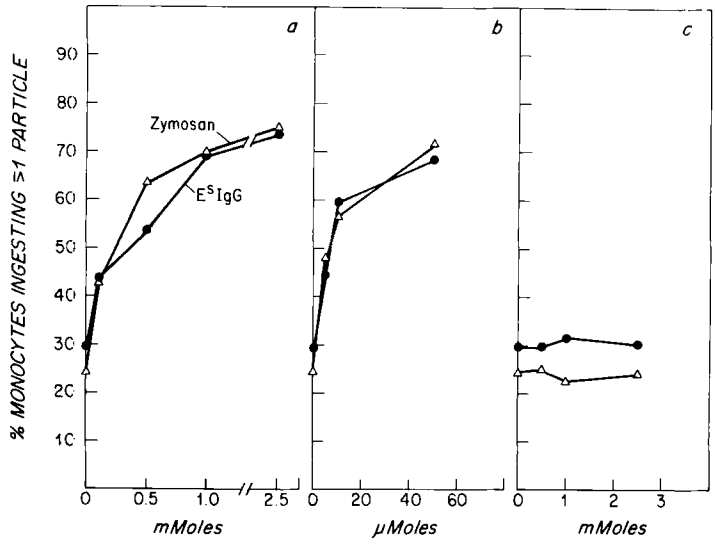


FIG. 2. Dose effects of Mg^{2+} (a), Co^{2+} (b), and Ca^{2+} (c) on monocyte ingestion of zymosan particles (△) and $E^{s}IgG$ (●). Adherent monocytes were incubated with target particles in GVB^{2-} containing various amounts of each cation per liter at 37°C for 30 minutes. The target-to-monocyte ratio was 10:1 and the data are the mean from two experiments.

shown in Fig. 2, GVB^{2-} supports low levels of zymosan and $E^{s}IgG$ ingestion involving 24 and 29% of the monocytes, respectively. The addition of Mg^{2+} increases monocyte ingestion in a dose-dependent fashion and with 0.5–1.0 m*M* Mg^{2+}, monocyte ingestion of both zymosan and $E^{s}IgG$ approaches maximal levels. The addition of Co^{2+} also results in dose-related increases in the ingestion of zymosan and $E^{s}IgG$; monocyte phagocytosis of both types of targets approaches maximal values with 10 μ*M*. At concentrations from 0.5 to 5 m*M*, Ca^{2+} has no effect on the ingestion of either type of target. Since ligand recognition by human monocyte Fc receptors is cation independent (Lobuglio *et al.*, 1967) and since monocyte phagocytosis of $E^{s}IgG$ and zymosan are comparably affected by these three cations, it is unlikely that the cation effects on monocyte ingestion of zymosan are at the recognition level. Instead, it would appear that cellular responses, activatable by Mg^{2+} or Co^{2+}, are necessary for maximum ingestion. Thus, these studies suggest that membrane-associated complement proteins of the alternative pathway are not the monocyte β-glucan receptor.

C. Cellular Responses

Monocyte recognition of unopsonized zymosan rapidly leads to ingestion and to the activation of other cellular responses. Relatively little has been reported about monocyte responses induced by unopsonized zymosan; how-

ever, some information is available concerning the release of extracellular lysosomal enzymes, production of oxygen metabolites, and generation of leukotrienes. The lysosomal enzyme, hexosaminidase, is secreted in small quantities by human monocytes incubated with unopsonized zymosan (Leoni and Dean, 1984). The zymosan-mediated release is not inhibitable but slightly stimulated by large concentrations of yeast α-mannans suggesting that a mannose receptor is not involved in hexosaminidase secretion. Others have shown that human monocytes lack mannose receptors (Shepherd *et al.*, 1982) comparable to those initially described with rat alveolar macrophages (Stahl *et al.*, 1978). These findings lend further support that a mannose receptor is not involved. The mannose receptor is present on a variety of macrophages and exhibits ligand specificity for mannosyl-, hexosamino-, and L-fucosyl-terminal residues of glycoproteins (Stahl *et al.*, 1978; Shepherd *et al.*, 1981). Since many lysosomal enzymes fall into this category, mannose receptors have been implicated in the transport of lysosomal enzymes. Human monocytes that have been cultured *in vitro* acquire mannose receptors (Shepherd *et al.*, 1982; Leoni and Dean, 1984) and secrete larger quantities of zymosan-inducible hexosaminidase than freshly explanted monocytes, but these levels are marginally reduced by large amounts of α-mannan (Leoni and Dean, 1984). These data suggest that β-glucan rather than α-mannan constituents of zymosan are the components which activate monocyte secretion of hexosaminidase.

The interaction of human monocytes and unopsonized zymosan particles produces oxygen metabolites. The generation of these products is initiated by a variety of transmembrane stimuli, which activate a cofactor-dependent oxidase located in plasma membranes; the activated oxidase has the capacity to reduce molecular oxygen to superoxide anions (O_2^-) (reviewed by Babior, 1984). Perturbation of monocyte plasma membranes by unopsonized zymosan particles results in oxidase activation and extracellular release of generated superoxide (Ross *et al.*, 1985). Superoxide anions are highly unstable and rapidly interact with other O_2^- molecules to produce hydrogen peroxide (H_2O_2). In the presence of the granule-associated enzyme, myeloperoxidase, hydrogen peroxide is a potent oxidizing agent. Numerous studies have revealed that in the presence of myeloperoxidase, H_2O_2 oxidizes chloride ions to hypochlorite (bleach); however, the myeloperoxidase–H_2O_2 system has the potential to oxidize other reactants as well. Of interest are the findings of Rosen and Klebanoff (1976) who demonstrated that in cell-free systems, unopsonized zymosan evokes a strong chemiluminescent response when incubated with myeloperoxidase and H_2O_2, suggesting that this response is mediated by the oxidation of zymosan, a particularly good substrate with its preponderance of oxidizable hydroxyl groups. Diamond and Haudenschild (1981) have shown that in the absence of serum, human monocytes recognize and ingest *Candida albicans*, a budding yeast that is

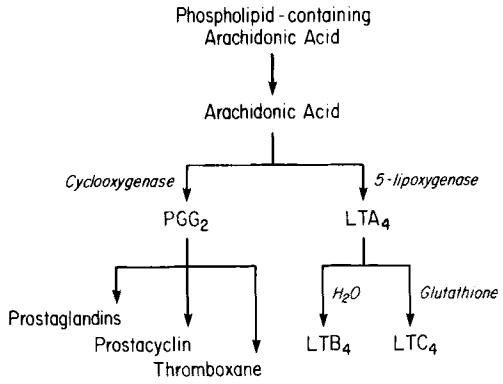


FIG. 3. Two pathways for the oxidation of arachidonic acid. Polyunsaturated arachidonic acid is released from plasma membrane phospholipids by phospholipases and then oxidized by enzymes of the cyclooxygenase or 5-lipoxygenase pathway.

structurally similar and composed of the same two glycans prominent in zymosan (Bishop *et al.*, 1960). Electron micrographs of the ingested yeast reveal cell wall damage which is largely attributed to oxidative mechanisms involving myeloperoxidase-catalyzed oxidations by H_2O_2. Such structural analyses of internalized yeast suggest that monocyte oxidative metabolites generally detected as extracellular products can potentiate intracellular damage of particles encased in phagosomes or phagolysosomes.

Perturbation of the β-glucan receptor with unopsonized zymosan particles or yeast glucan particles activates monocyte metabolism of endogenous arachidonic acid. Arachidonic acid is the major fatty acid constituent of membrane phospholipids (Stossel *et al.*, 1974) and is released by phospholipases during transmembrane activation. As shown in Fig. 3, released arachidonic acid is oxidized through one of two pathways. Enzymes of the cyclooxygenase pathway convert it to prostaglandins, prostacyclin, or thromboxane; enzymes of the 5-lipoxygenase pathway generate leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4). Phagocytic stimulation of the monocyte Fc-IgG receptor is more effective than that of the β-glucan receptor in the release of cyclooxygenase products (Passwell *et al.*, 1979), whereas perturbation of the β-glucan receptor preferentially activates the 5-lipoxygenase pathway (Williams *et al.*, 1984). Monocyte phagocytic and leukotriene responses initiated by unopsonized zymosan particles follow the same time course, exhibit the same sensitivity to trypsin inactivation, the same dose-inhibition by β-glucan, and the same indifference to α-mannan (Williams *et al.*, 1984; Czop and Austen, 1985b). Since the trypsin effects do not involve inactivation of the 5-lipoxygenase enzymes, the leukotriene response is activated through the trypsin-sensitive β-glucan receptor. The incubation of yeast glucan particles with human monocytes promotes a comparable pha-

gocytic response but a more efficient leukotriene response than that seen with similar inputs of zymosan particles. As with zymosan, activation of phagocytosis and leukotriene generation by yeast glucan particles are both inhibited by β-glucans and unaffected by α-mannans (Czop and Austen, 1985b). Thus, a common monocyte β-glucan receptor is activatable by either zymosan or yeast glucan particles to produce leukotrienes. Since all of the generated leukotrienes are quickly released from cells, it is unlikely that these products have any effects on ingested particles. However, these lipoxygenase products have the capacity to potentiate and amplify inflammatory responses. The major lipoxygenase product released after activation of the β-glucan receptor is LTB_4, which is a potent chemotactic factor (Borgeat and Samuelsson, 1979; Goetzl and Pickett, 1980; Smith *et al.*, 1980) and which promotes adherence of neutrophils to endothelial cell surfaces (Dahlén *et al.*, 1981).

III. Phagocytosis of Unopsonized Zymosan by Nonelicited Murine Macrophages

In the absence of serum, nonelicited and thioglycollate-elicited murine peritoneal macrophages ingest unopsonized zymosan particles (Rabinovitch and DeStefano, 1973). The phagocytic response of cells from thioglycollate-treated mice is initially slightly less than that for cells from untreated mice but increases with culture. The initial decrease may be due to constituents in the thioglycollate broth which contains 100–500 μg/ml of thioglycollate and 5 mg/ml of yeast extract. A variety of eliciting agents are used to increase yields of peritoneal macrophages and to study differences between these macrophages and resident cells. In this review, discussion is limited to nonelicited cells because it is sometimes difficult to evaluate resident and elicited cells with respect to some ligand–receptor interactions. Most noteable are the responses regulated by mouse CR1 and/or CR3 receptors, which recognize mouse but not human C3 fragments (Bianco *et al.*, 1970; Dierich *et al.*, 1974). Since purification of mouse C3 (Kinoshita and Nussenzweig, 1984) is appreciably more laborious than human C3, mouse C3 fragments are generally deposited on E^sIgM by incubations in C5-deficient mouse sera. Adsorption of additional serum proteins during this deposition is possible. The presence of small quantities of additional components would be less likely to affect nonelicited than elicited cells, which are generally more responsive to most stimulating agents.

A. Characterization of the Phagocytic Receptor

Opsonin-independent phagocytosis of zymosan particles by resident peritoneal macrophages has been distinguished from Fc-mediated responses in several different ways. Polyclonal antibodies raised to murine macrophage surface determinants block ingestion of E^sIgG but not unopsonized zymosan

(Holland *et al.*, 1972). The mouse receptor which initiates zymosan ingestion continues to function even though interference of the Fc-mediated phagocytic response involves clearance of the Fc receptors and of at least one other membrane constituent. Macrophage receptors for Fc-IgG and unopsonized zymosan are also distinguishable by their interactions with 2-deoxyglucose (Michl *et al.*, 1976a). Preincubation of normal murine peritoneal macrophages with 50 m*M* of 2-deoxyglucose reduces their subsequent capacity to ingest E^{s}IgG but not zymosan particles. The mechanism by which the Fc receptor is functionally inhibited appears to involve membrane-related events independent of glycolysis (Michl *et al.*, 1976b).

Several studies have been presented concerning the glycan specificity of the murine phagocytic receptor for unopsonized zymosan particles. Identification of mannose receptors on nonelicited mouse peritoneal macrophages (Ezekowitz *et al.*, 1981) has focused attention on the α-mannan constituent of zymosan. Dose–response experiments have been carried out with various types of α-mannans in which mouse peritoneal macrophages are pretreated and maintained during zymosan ingestion; a 50% reduction in zymosan phagocytosis occurs with a mean concentration of 5 mg/ml of *S. cerevisiae* α-mannans (Sung *et al.*, 1983). This concentration is comparable to that which gives 50% reductions with human monocytes, namely 7.6 mg/ml of α-mannon that has not been pretreated with β-glycosidases (Czop and Austen, 1985a). When β-glucans are included in studies with murine macrophages, the ligand specificity of the mouse phagocytic receptor for unopsonized zymosan is equivalent to that of human monocytes. Choi and Kadish, using assays based on the functional properties of monocyte β-glucan receptors, have studied phagocytic responses of murine macrophages cultured from bone marrow. In the absence of serum, these macrophages avidly ingest E^{s}IgG, unopsonized zymosan particles, and yeast glucan particles. Preincubation of murine macrophages with barley β-glucan or laminarin reduces their subsequent capacities to ingest zymosan particles but not E^{s}IgG; yeast α-mannan at 1 mg/ml has no effect on the phagocytosis of either target (Fig. 4). In the absence of glycan, essentially all of the macrophages ingest between 1 and 5 particles, 53% ingest between 6 and 9, and half of these (27%) ingest 10–25 zymosan particles. Yeast α-mannan does not change these percentages, but barley β-glucan or laminarin reduces the numbers of macrophages ingesting $\geq$ 6 zymosan particles by about 60% and those ingesting $\geq$ 10 zymosan particles by nearly 100%. Thus, in terms of ligand specificity, the murine receptor which initiates phagocytosis of unopsonized zymosan is appropriately designated the β-glucan receptor.

B. Cellular Responses

To illustrate murine responses activatable by unopsonized zymosan particles, two of the three areas discussed for human monocytes are covered:

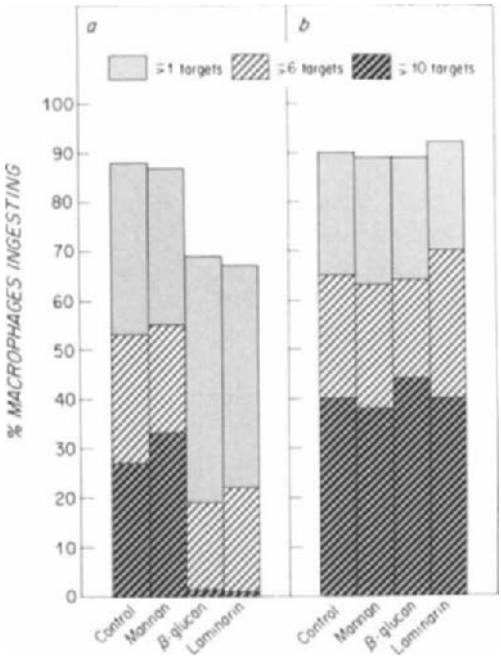


FIG. 4. Effects of yeast α-mannan, barley β-glucan, and laminarin on phagocytosis by cultured bone marrow-derived mouse macrophages. Monolayers of macrophages were pretreated at 37°C for 20 minutes, washed, and assessed for their phagocytosis of 1.25×10^7 zymosan particles (a) and E^sIgG (b) after a 30-minute incubation. The data are expressed as the percentages of macrophages ingesting ≥ 1, ≥ 6, and ≥ 10 targets and are the mean of four experiments. Macrophages pretreated with 100 μg/ml of barley β-glucan or laminarin exhibited phagocytic capacities comparable to those shown.

lysosomal enzymes and arachidonic acid metabolites. Nonelicited mouse peritoneal macrophages produce little superoxide or hydrogen peroxide regardless of stimulus (Nathan and Root, 1977; Johnston *et al.*, 1978). Most of the oxygen consumed by phagocytosing resident mouse macrophages undergoes oxidative phosphorylation and is not converted to superoxide or H_2O_2. It is of interest that when human monocytes are cultured, they begin to resemble murine macrophages with regard to oxygen consumption and generate almost no superoxide or H_2O_2 (Nakagawara *et al.*, 1981).

Schnyder and Baggiolini (1978) have presented an elegant series of studies with nonelicited mouse peritoneal macrophages and various enzymes activatable by unopsonized zymosan particles. Macrophages which have phagocytosed zymosan are washed and assessed for various cellular enzymes during subsequent culture for up to 10 days. Immediately after zymosan ingestion, intracellular levels of two lysosomal enzymes, hexosaminidase and

β-glucuronidase, are rapidly reduced and these decreases are accompanied by corresponding increases in secreted products. During short-term culture, both of these hydrolases continue to be released and reach levels 1–2 times of those originally detected within the cells. The intracellular levels also increase and plateau at their original levels. Thus, zymosan activates release of the entire intracellular supply of both enzymes and does not inhibit restoration to physiologic intracellular levels. In the absence of further zymosan stimulation, longer term cultures progressively increase their intracellular levels of lactic acid dehydrogenase (LDH), extracellular release of lysozyme, and generate substantial quantities of plasminogen activator. Thus, following zymosan ingestion, murine macrophages exhibit a time-dependent sequence of events: release and restoration of lysosomal enzymes, increased intracellular LDH, and release of lysozyme and plasminogen activator.

A number of cellular responses are initiated by zymosan immediately after its recognition by the murine β-glucan receptor. Bonney *et al.* (1978) have developed methods for incorporating radiolabeled fatty acids into murine lipids, thereby permitting study of lipid metabolism. The only cellular lipid affected by zymosan-induced phagocytic responses is arachidonic acid which is oxidized to products of the cyclooxygenase (Bonney *et al.*, 1978; Scott *et al.*, 1980) and 5-lipoxygenase (Rouzer *et al.*, 1980a,b) pathways. Zymosan rapidly initiates synthesis and release of the cyclooxygenase product prostaglandin E_2 before release of lysosomal hexosaminidase is detected. Chromatographic analysis for cyclooxygenase products reveals several prostaglandins and thromboxane (Bonney *et al.*, 1978). Using techniques which permit separation of cyclooxygenase and 5-lipoxygenase products, Rouzer *et al.* (1980a,b) have calculated that approximately 67% of the released arachidonate metabolites are cyclooxygenase products and 15–30% is the 5-lipoxygenase product, leukotriene C (LTC). After incubation with zymosan particles, macrophages rapidly generate LTC and PGE_2 at comparable rates which are time and dose dependent on the phagocytic response initiated by zymosan (Rouzer *et al.*, 1980b; Scott *et al.*, 1980). Thus, activation of arachidonate metabolism is one the first cellular responses induced by macrophage phagocytosis of zymosan.

Ingestion through murine Fc-IgG receptors also initiates synthesis and release of cyclooxygenase products and LTC. After stimulation of Fc-IgG receptors, cyclooxgenase products are generated at concentrations comparable to those inducible by stimulation of β-glucan receptors with unopsonized zymosan; however, Fc-mediated induction results in the release of 3- to 10-fold less LTC than that by zymosan (Rouzer *et al.*, 1980b, 1982). Subsequent identification of the 5-lipoxygenase products generated by zymosan-activated macrophages reveals leukotrienes C_4, D_4, and E_4 (reviewed by Lewis *et al.*, 1982). These leukotrienes are the major constituents of slow-releasing

substance A (SRS-A) which is a potent lipid mediator in sites of acute and chronic inflammation. The capacity of macrophages to generate substantial amounts of these lipid mediators in the absence of opsonins indicates that the β-glucan receptor represents an important physiologic mechanism for their production.

A final thought is appropriate in closing the discussions about unopsonized zymosan particles. Latex beads and zymosan are often lumped together as examples of nonspecific phagocytosis. To put latex in perspective, the following information is provided. Latex beads do not activate the alternative complement pathway (Czop *et al.*, 1978a). In the absence of serum, latex beads do not activate production and/or release of cellular enzymes (Axiline and Cohn, 1970; Schorlemmer *et al.*, 1977; Mørland and Kaplan, 1977; Schnyder and Baggiolini, 1978) and do not activate oxidative metabolism of arachidonic acid (Glatt *et al.*, 1977; Bonney *et al.*, 1978). Monocytes pretreated with trypsin associate with as many latex beads as untreated cells (Czop *et al.*, 1978a) and viable monocytes/macrophages are essentially impossible to remove from latex plastic plates with trypsin. Thus, the incubation of latex beads with macrophages or monocytes already plated on the same kinds of plastic fails to stimulate any biochemical event associated with the attachment and ingestion of phagocytosed particles.

IV. Phagocytosis of Nonmicrobial Particulate Activators by Human Monocytes

A. Characterization of the Phagocytic Receptor

In the absence of serum, human monocytes ingest unopsonized erythrocytes from rabbits (E^r), from two species of mice (E^m), but not those from sheep (E^s) and guinea pigs (E^{gp}). The capacity of E^r and E^m to initiate a monocyte phagocytic response directly parallels their capacity to activate the plasma proteins of the human alternative complement pathway; E^s and E^{gp} do not activate this pathway (Czop *et al.*, 1978a). Quantitative removal of sialic acid residues or chemical modification of E^s progressively converts these particles from nonactivating particles to activators of the phagocytic response (Czop *et al.*, 1978b) and of the alternative pathway (Fearon, 1978; Pangburn and Müller-Eberhard, 1978). Monocyte ingestion of E^r is greater than that of desialated E^s but ingestion of both is reduced in a comparable dose-dependent fashion by the same low concentrations of trypsin that reduce monocyte ingestion of zymosan (Czop *et al.*, 1978b). As with zymosan, phagocytosis of E^r and desialated E^s by trypsin-treated cells is fully reexpressed within 24–48 hours of culture in serum-free media. In the absence of trypsin pretreatment, cultured monocytes continue to exhibit the capacity to ingest both erythrocyte activators for at least 48 hours (Czop and Austen,

1980). Thus, a common monocyte receptor initiates phagocytosis of at least two non-microbial particulate activators, E^r and desialated E^s.

Little is known about the chemical composition or structure of E^r membranes; they contain 10-fold fewer terminal sialic acid residues than E^s membranes (Aminoff *et al.*, 1976). However, monocyte ingestion of E^r is inhibited to the same extent and by the same glycans which inhibit zymosan ingestion (Czop and Austen, 1985a). As shown in Table I, of the four β-glucans with 1,3 and/or 1,6 linkages, laminarin is the most efficient inhibitor of E^r ingestion. Cellulose, α-glucans, galactans, and mannans are each non-inhibitory. Thus, monocyte phagocytosis of nonmicrobial (E^r) and microbial (zymosan) particulate activators is initiated by a common β-glucan receptor which exhibits the same trypsin sensitivity, ligand specificity, and culture responsiveness.

Relative to zymosan, E^r are ligand-limiting particles in the alternative pathway and in monocyte ingestion. In assays developed for titering alternative complement proteins, E^r utilize at least 10-fold less B than zymosan, and in phagocytic assays they initiate ingestion of only some of the monocytes which ingest zymosan. The numbers of monocytes ingesting E^r vary among different donors but remain relatively constant for a given donor. Over an 8-year period, routine retesting of monocytes obtained from 40 different donors revealed no significant donor-dependent changes in the numbers of cells ingesting E^r. Donors from a normal population have been operationally grouped as low, intermediate, and high ingesters largely on the basis of their capacities to ingest E^r. By other criteria, monocytes isolated from the three types of ingesters also exhibit quantitative differences. In dose–response experiments with E^r, monocytes from all three donor types exhibit a dose-dependent phagocytic increase which is followed by a dose-dependent decrease (Czop *et al.*, 1978a), suggesting that an individual E^r must occupy a critical number of β-glucan receptors for ingestion. The dose at which E^r begin to inhibit ingestion and the extent of inhibition differ with monocytes isolated from the three ingester types (Czop *et al.*, 1978a). At inputs of from 50 to 150 E^r/monocyte, the percentages of cells ingesting E^r increase from 5–12, 15–40, and 20–70% for monocytes isolated from low, intermediate, and high donor ingesters, respectively. Monocytes from low ingester donors are most susceptible to inhibition by increased numbers of E^r, whereas those from intermediate and high ingesters are progressively less susceptible to being overwhelmed by large inputs of E^r. These data suggest that ingester status is related to the number of functional β-glucan receptors on donor monocytes.

The distribution of β-glucan receptor function has been examined for monocytes from a normal adult population. As shown in Table III, most individuals are low ingester donors and only 12% are high ingesters. Family

TABLE III
DISTRIBUTION OF β-GLUCAN RECEPTOR FUNCTION ON MONOCYTES FROM A NORMAL HUMAN DONOR POPULATION[a]

Type of donor	Donors (%)
Low	68
Intermediate	20
High	12

[a] Monocytes were isolated from 200 different donors and assessed in duplicate for their ingestion of 1–2 × 10^8/ml and of 1 × 10^9/ml E^r in serum-free media. Monocytes from known low and high ingester donors were used as a reference in each group of experiments. At inputs of 20–50 unopsonized zymosan particles per monocyte, all monocytes ingest zymosan, regardless of donor origin.

studies have been carried out with two families. With the first family, monocytes were screened from the mother, father, and four siblings whose ages ranged from 27 to 34: each of these individuals presented as low ingester donors. With the second family, monocytes were screened from the mother, father, and four siblings between the ages of 19 and 28. All of the members of this family were screened twice except for the mother. On the second occasion, each of the 6 individuals was typed for histocompatibility antigens by the laboratories of Dr. Edmond J. Yunis, Sidney Farber Cancer Institute, Boston. The distribution of β-glucan receptor function on monocytes from this family is shown in Fig. 5. Monocytes from the mother, father, and two related siblings tested as intermediate ingesters when compared to unrelated reference donors of low and intermediate types. Monocytes from one brother presented as low and those from another brother as high. All three brothers, the low, intermediate, and high, exhibited identical histocompatibility antigens as assessed with 54 antisera against HLA-A, B, C, D, and Dr antigens. Thus, β-glucan receptor function is genetically controlled and unrelated to antigens of the HLA system.

B. CELLULAR RESPONSES

Unlike monocyte cellular responses activatable by unopsonized zymosan particles, there is no apparent literature concerning cellular processes induced by the phagocytosis of unopsonized nonmicrobial particulate activators.

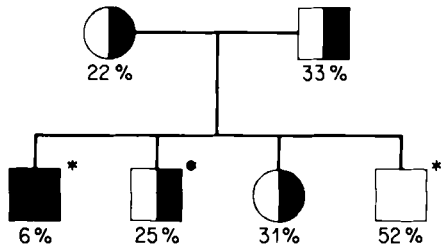


FIG. 5. Distribution of β-glucan receptor function on monocytes from one family. Monocytes from 6 family members, an unrelated low ingester donor, and an unrelated intermediate ingester were isolated and assessed in duplicate for their phagocytosis of 3×10^6/ml unopsonized E^r. The percentage of monocytes ingesting ≥ 1 E^r is indicated below the symbol for each family member. Symbols that are closed, half-closed, and open depict low, intermediate, and high ingesters, respectively. The percentages of monocytes ingesting ≥ 1 E^r from the unrelated low and intermediate ingesters were 7 and 25%, respectively. The asterisk (*) indicates identical histocompatibility serotypes.

V. Phagocytosis of Nonmicrobial Particulate Activators by Nonelicited Murine Macrophages

A. Characterization of the Phagocytic Receptor

In the absence of serum, nonelicited murine peritoneal macrophages ingest large numbers of unopsonized guinea pig, frog, and chicken thymocytes but not mouse thymocytes (Sano *et al.*, 1980). The greatest phagocytic response is initiated by chicken thymocytes which stimulate 65–95% of the murine macrophages to ingest ≥ 3 of these targets. The mechanism by which macrophages phagocytose chicken thymocytes has been examined in terms of their activating the mouse alternative complement pathway (Sugimoto *et al.*, 1980). Chicken thymocytes induce cleavage of mouse C3 and deposition of C3 fragments on their surface when they are incubated with normal mouse serum. Sera functionally depleted by zymosan, chelated with EDTA, or inactivated by heat treatment do not support deposition of C3b fragments on chicken thymocytes. Thus, chicken thymocytes are activators of the mouse alternative complement pathway.

The macrophage receptors that mediate ingestion of unopsonized chicken thymocytes have been functionally distinguished from murine receptors for Fc-IgG. Pretreatment of macrophages with immune complexes blocks Fc-mediated ingestion of E^sIgG but has no effect on the ingestion of unopsonized chicken thymocytes (Sugimoto *et al.*, 1980). Macrophage recognition of unopsonized chicken thymocytes rapidly leads to ingestion without an apparent attachment phase. It is of interest that although homologous thymocytes are not ingested, they become firmly attached to murine mac-

rophages (Sano *et al.*, 1980). When chicken thymocytes are opsonized with normal mouse serum to permit generation and deposition of mouse C3 fragments, these targets adhere firmly to the macrophage membrane and promote slightly higher levels of ingestion (Sugimoto *et al.*, 1980). Thus, the presence of mouse C3 fragments on chicken thymocytes markedly stimulates their attachment to mouse C3 receptors.

Phagocytosis of unmodified heterologous erythrocytes have not been reported for murine macrophages under serum-free conditions. E^r activate the mouse alternative complement pathway but this capacity varies and is dependent on mouse strain and sex (van Dijk *et al.*, 1980). The length of time that E^r are stored decreases their capacity to activate mouse complement and may explain why studies with murine macrophages and E^r have not been reported.

B. Cellular Responses

Nonelicited murine peritoneal macrophages phagocytose unopsonized heterologous erythrocytes that have been modified by glutaraldehyde (Rabinovitch, 1967) and formaldehyde (Schnyder and Baggiolini, 1978). Both of these aldehydes are strong reducing agents. Capo *et al.* (1979) have demonstrated that nonelicited rat peritoneal macrophages ingest a variety of aldehyde-treated erythrocytes under serum-free conditions. In their studies on murine enzymes, Schnyder and Baggiolini (1978) compared the capacities of unopsonized zymosan and formaldehyde (formalin)-treated E^s to activate murine macrophages. In the absence of opsonins, phagocytosis of formalin-treated E^s activates synthesis and/or release of enzymes in the same time-related fashion as that observed with zymosan (Section III,B). In their discussion, Schnyder and Baggiolini (1978) comment that formalin-treated E^s are not activators of the alternative complement pathway; however, the methods by which this capacity was measured and the species of alternative pathway proteins evaluated were not reported.

VI. Particulate Activators of the Alternative Complement Pathway

Cleavage of C3 through the alternative complement pathway is promoted by a variety of substances among which are zymosan (Pillemer *et al.*, 1954), yeast glucan particles (Seljelid *et al.*, 1981), pachyman (Hamuro *et al.*, 1978), insolubilized laminarin (Seljelid *et al.*, 1981), nigeran (Inai *et al.*, 1976), an α-glucan synthesized enzymatically from sucrose by filtrates of *Streptococcus mutans* (Inai *et al.*, 1976), lipopolysaccharides (LPS) of gram-negative bacteria (Gewurz *et al.*, 1968), inulin (Götze and Müller-Eberhard, 1971), and E^r (Platts-Mills and Ishizaka, 1974). Although this list is incomplete, the activating components of particulate activators are very often carbohydrates

which contain repeating subunits. Zymosan and yeast glucan particles are essentially pure carbohydrates, as are pachyman (Warsi and Whelan, 1957), laminarin (Black *et al.*, 1951), nigeran (Johnston, 1965), the microbial α-glucan (Inai *et al.*, 1976), and inulin (Bacon and Edelman, 1951), a fructan extracted from the tubers of Jerusalem artichokes. The constituents of LPS which activate the alternative pathway are the polysaccharide side chains (Morrison and Kline, 1977) and those of E^r are unknown.

The glycans used to define the ligand specificity of the monocyte β-glucan receptor have been assessed for their capacities to activate the human alternative complement pathway (Czop and Austen, 1985c). The results of these studies reveal several important features about activators and are summarized in Table IV. On a weight basis, yeast glucan particles and pustulan, a β-D-(1,6)-glucan (Lindberg and McPherson, 1954), are more potent activators than zymosan, suggesting that the active constituents of zymosan are its β-(1,3) and/or β-(1,6) glucans, rather than its mannan components. All of

TABLE IV
CAPACITY OF VARIOUS GLYCANS TO ACTIVATE THE HUMAN ALTERNATIVE COMPLEMENT PATHWAY[a]

Glycan	Predominant chemical composition	Concentration (μg/ml) for 50% activation
Laminarin	β-D-Glucose (1,3; 1,6)	—[b]
Barley β-glucan	β-D-Glucose (1,3 and 1,4)	60
Pachyman	β-D-Glucose (1,3)	350
Pustulan	β-D-Glucose (1,6)	27
Cellulose	β-D-Glucose (1,4)	NA[c]
Sephadex G-25	α-D-Glucose (1,6)	NA
Nigeran	α-D-Glucose(1,3 and 1,4)	500
Agarose	α-L/β-D-Galactan (1,3 and 1,4)	NA
Mannan	α-D-Mannose (1,6; 1,2)	—
Inulin	β-D-Fructose (2,1)	
Pyrogen-free		—
Crude		700
Crude-purified		NA
Zymosan particles	β-D-Glucose (1,3 and 1,6; 1,3)/mannan	47
Glucan particles	β-D-Glucose (1,3 and 1,6; 1,3)	23

[a] Normal human serum was incubated at 1:2 dilutions with glycans, 8 m*M* EGTA, and 2 m*M* $MgCl_2$ at 37°C for 30 minutes, clarified by centrifugation, and the supernatants assessed for residual lysis of E^r at final serum dilutions of 1:8. The classical complement pathway was not activatable under these conditions.

[b] Soluble glycan preparations.

[c] NA, nonactivator at ≥2 mg/ml.

the particulate β-glucans with 1,3 and/or 1,6 linkages are more active than the α-glucan with 1,3 linkages (nigeran); the α-glucan with 1,6 linkages (Sephadex) is a nonactivator (Inai *et al.*, 1976, Table IV). The data suggest that the β-anomeric configuration of glucose is more optimal for alternative pathway activation than the α-anomeric one. Crude inulin is the least potent activator and purified insoluble inulin does not activate the pathway, suggesting that the β-D-fructose composition is unlikely to present an activating surface. Two additional points deserve mention. First, overall structure is more important for activation than monosaccharide composition. This is most easily illustrated with cellulose, a linear β-glucan with 1,4 linkages and a nonactivator. Second, the capacity of a glycan to activate the alternative complement pathway is dependent on its chemical structure and its physical state of aggregation. Soluble laminarin does not activate the alternative pathway (Table IV), but insoluble laminarin does (Seljelid *et al.*, 1981). Conversely, modification of the insoluble activator, pachyman, to more soluble forms results in a loss of alternative pathway activity (Hamuro *et al.*, 1978), and sedimentation of barley β-glucan and pustulan removes micro- and/or macroaggregates necessary for alternative pathway activation even though the resulting supernatants contain significant amounts of high molecular weight β-glucans (Czop and Austen, 1985c). Thus, β-glucans with consecutive or branched linkages prominent in yeast glucan and zymosan particles are potent activators of the human alternative complement pathway. The term particulate becomes especially appropriate since soluble forms of these β-glucans do not activate.

VII. Effects of Fibronectin

A. Structure of Fibronectin

Plasma fibronectin is a large glycoprotein which is present in normal human plasma in concentrations of 150–550 μg/ml and was initially described by Morrison *et al.* (1948) as cold-insoluble globulin. It has a molecular weight of 440,000 (Mosesson and Umfleet, 1970), is composed of two similar but nonidentical polypeptide chains with estimated molecular weights of 220,000 and 210,000 (Mosesson *et al.*, 1975) that are disulfide-linked at the carboxy-end of the molecule (Furie and Rifkin, 1980). The larger (α) and shorter (β) chains of human plasma fibronectin consist of a series of similarly ordered domains: an NH_2-terminal domain of 27,000–29,000 (Furie *et al.*, 1980; Mosher and Proctor, 1980), an adjacent gelatin-binding domain of 40,000 (Furie *et al.*, 1980), and a complex region of 160,000 and 140,000 from the α- and β-chains, respectively (McDonald and Kelley, 1980; Ehrismann *et al.*, 1982). Each of these latter high-molecular-

weight fragments contains three domains: a cell-adhesive domain of 80,000–100,000, within which is a 12,000–15,000 molecular weight peptide that mediates attachment and/or spreading of chicken myoblasts (Ehrismann *et al.*, 1982) and rat kidney cells (Pierschbacher *et al.*, 1981); a domain of 30,000–40,000 which mediates high-affinity heparin-binding (Richter and Hörmann, 1982); and a domain of 30,000–37,000 which is adjacent to the interchain-disulfide bonds (Richter and Hörmann, 1982).

Protein sequence data for several fibronectin domains reveal three types of repeating homologous units, each of which contains 50–90 amino acid residues (Skorstengaard *et al.*, 1982; Petersen *et al.*, 1983). The NH_2-terminus consists of five looped units of type I homology which contains two intrachain disulfide bonds per unit. A region with three looped units of type I homology is also present within the carboxy-portion of the intact molecule. The gelatin-binding domain is heterologous in that it consists of one type I unit and two looped type II units. Sequences of type III homology lack intrachain disulfide bonds, are repeated at least four times near the middle of the fibronectin molecule, and may give rise to a polyvalent cell-adhesive domain.

Further structural analysis of fibronectin with functionally defined fragments is limited by the absence of any known binding or biological activities for at least half of the intact molecule and is complicated by the occurrence of the same functional marker at different regions of the intact fibronectin molecule. Although a number of monoclonal antibodies to human plasma fibronectin have been described, most of these have not been used as immunologic sequence probes. Some have been used to block fibronectin mediated attachment of fibroblasts to tissue culture plates (Pierschbacher *et al.*, 1981; Schoen *et al.*, 1982). Others have been mapped to one of many smaller fragments generated after extensive cleavage of larger positive fragments (Smith and Furcht, 1982; Dziadek *et al.*, 1983). By immunoblot analyses with several large overlapping fragments, Czop *et al.* (1985a) have mapped each of 5 monoclonal antibodies to a single defined region within the intact fibronectin molecule. One of these, monoclonal BC7, recognizes intrachain disulfide-formed determinants within the NH_2-terminal domain, whereas another, monoclonal AB3, recognizes intrachain disulfide-formed determinants within 35,000 Da of the carboxy-terminus. Although the regions recognized by these two monoclonals both contain sites susceptible for transamidation by Factor $XIII_a$ (Jilek and Hörmann, 1977; Richter *et al.*, 1981), sites for binding fibrin (Seidl and Hörmann, 1983; Sekiguchi and Hakomori, 1983), and amino acid sequences of type I homology, these regions do not contain epitopes for both BC7 and AB3. A third monoclonal, designated CE9, recognizes epitopes within a fibronectin fragment with a molecular weight of 18,000. This fragment links the gelatin-binding domain to the cell-

adhesive domain. Monoclonal CE9 has permitted the exact placement of this previously described fragment (Ehrismann *et al.*, 1982; Seidl and Hörmann, 1983) and provides a convenient amino-terminal marker for studying epitopes recognized by monoclonals with specificity for the cell-adhesive domain. Among these monoclonals is the one designated BD4, which is within 79,000 Da of the gelatin-binding domain. The epitope recognized by the fifth monoclonal, CPG1, is immediately adjacent to the interchain-disulfide bonds at the carboxy-terminus of the intact molecule, a region particularly sensitive to protease cleavage. The epitopes recognized by each of these monoclonals are within both chains of the intact molecule except the AB3 determinant, which is apparently present only on the α-subunit chain. These five monoclonal antibodies provide a defined framework within which additional immunologic probes can be precisely mapped to confirm or modify structural models of fibronectin.

B. Opsonic Effects of Intact Fibronectin

The capacity of fibronectin to function as an opsonin was initially described by Saba *et al.* (1966) who showed that a plasma protein termed opsonic α_2-globulin protein or opsonic α_2-surface binding glycoprotein promoted uptake of gelatin-containing lipid emulsions by rat liver slices. Blumenstock *et al.* (1978b) later demonstrated that this protein is identical to plasma fibronectin. Both proteins have molecular weights of 440,000, comparable amino acid compositions, and identical antigenic determinants.

The opsonic effects of intact plasma fibronectin have generally been studied with four test particles: E^s, formalin-treated E^s, modified latex beads, and lipid emulsions. In the absence of gelatin, intact fibronectin does not bind to any of these test particles. Gelatin-coated E^s (E^s-gel) have been used to identify fibronectin receptors on human monocytes (Bevilacqua *et al.*, 1981). In the presence of human plasma fibronectin, E^s-gel bind to monocytes but are not ingested (Bevilacqua *et al.*, 1981; Czop and Austen, 1982). The region within gelatin-bound human fibronectin which is recognized by human monocyte fibronectin receptors has been mapped with monoclonal antibodies to the cell-adhesive domain (Czop *et al.*, 1985a). Gelatin-coated formalin-treated E^s have been used to identify fibronectin receptors on rat peritoneal macrophages; fibronectin promotes attachment and augments ingestion of these targets (Rourke *et al.*, 1984). The fibronectin receptors on rat macrophages recognize fragments of rat (Rourke *et al.*, 1984) and human (Molnar *et al.*, 1983) plasma fibronectin that lack NH_2-terminal domains and carboxy-terminal regions of the intact molecules. In the presence of fibronectin, gelatin-conjugated latex beads are taken up by rodent macrophages by a mechanism which is heparin dependent (Gudewicz *et al.*, 1980; van de Water *et al.*, 1981) or heparin enhanced (Doran *et al.*, 1981a). Gelatin and

heparin are required cofactors for fibronectin-dependent uptake of lipid emulsions by rat liver slices (Molnar *et al.*, 1977; Blumenstock *et al.*, 1978a), but these test particles do not appear to be ingested (Molnar *et al.*, 1979). Thus, with each of these four test particles, fibronectin exhibits no opsonic function until the targets are "opsonized" with gelatin. If the gelatin-coated target lacks an independent phagocytic signal, fibronectin promotes adherence to fibronectin receptors without phagocytosis. There is one exception. In the absence of gelatin or heparin, Pommier *et al.* (1983) have reported that intact human fibronectin enhances Fc-mediated ingestion and alters monocytes to phagocytose through CR1. The mechanism by which soluble fibronectin mediates these effects is largely unknown but appears to involve fibronectin interactions with subsets of human monocytes.

In contrast to these test particles, intact human plasma fibronectin binds directly to a variety of live microorganisms in the absence of cofactors. Human fibronectin has been demonstrated to bind to protein A-containing strains of *Staphylococcus aureus* (Doran and Raynor, 1981), clinical strains of S. *epidermidis* and protein A-deficient S. *aureus* (Verbrugh *et al.*, 1981), *Streptococcus pneumoniae* (Hof *et al.*, 1980), some strains of *Pseudomonas aeruginosa* (Niehaus *et al.*, 1980), virulent forms of *Treponema pallidum* (Peterson *et al.*, 1983), and viral envelop glycoproteins from influenza, parainfluenza, and mumps viruses (Julkunen *et al.*, 1983). The opsonic value of these fibronectin interactions is difficult to assess since subsequent incubation of some of these fibronectin-coated microbes with various human phagocytes promotes relatively little phagocytosis (Verbrugh *et al.*, 1981; Doran *et al.*, 1981b).

C. Opsonic Effects of a 180,000 M_r Fibronectin Fragment

The idea that a fragment of fibronectin could act as an opsonin without gelatin or heparin cofactors came from two findings. First, some but not all preparations of gelatin-purified human plasma fibronectin markedly enhance monocyte phagocytosis in the absence of cofactors. Second, purified intact human plasma fibronectin which is phagocytically inactive acquires enhancing activity after limited protease cleavage (Czop *et al.*, 1981). Two distinctly different monoclonal antibodies to intact human plasma fibronectin, BD4 and AB3, were selected to purify the active constituent (Czop *et al.*, 1981, 1982a). Monoclonal BD4 recognizes the intact molecule in solution and immobilized on polyvinyl chloride plates or on nitrocellulose paper. Monoclonal AB3 recognizes immobilized but not soluble intact fibronectin. Affinity reagents prepared with either of these monoclonal antibodies retain the active components generated from intact fibronectin by protease cleavage.

By sequential affinity chromatography, a single protein with a molecular

weight of 180,000 has been isolated from human plasma (Czop *et al.*, 1982a). On a weight basis, this protein is about 100–200 times more active than the gelatin-derived fibronectin preparations; after reduction, it presents as three proteins with molecular weights of 82,000, 52,000, and 27,000, suggesting that the protein contains disulfide-linked polypeptides. By Ouchterlony analysis, the 180,000 molecular weight protein exhibits partial identity with intact human plasma fibronectin indicating that it lacks some of the determinants present in the intact molecule. At 175 μg/ml, the purified protein forms no precipitin lines with antisera to whole human serum, to five proteins of the alternative complement pathway, to the five classes of immunoglobulins, to several classical pathway complement proteins, to fibrinogen, and to various α_1- and α_2-globulins. It contains a gelatin-binding domain and at least four epitopes of human plasma fibronectin. This opsonic fibronectin fragment has been designated as 180K-opFnf to indicate its molecular weight, function, and parent molecule.

The opsonic function of 180K-opFnf is selective for the phagocytic responses mediated by β-glucan receptors because it directly binds to particulate activators but not to nonactivators such as E^s (Czop *et al.*, 1981, 1982a). In the absence of serum or cofactors, 180K-opFnf augments ingestion of the particulate activators, zymosan, E^r, and E^m, in a dose-related fashion, but does not affect monocyte Fc-mediated ingestion of E^sIgG or induce ingestion of E^s or E^sC3b. Concentrations of 180K-opFnf as low as 0.5–1.0 μg/ml can increase the percentage of monocytes ingesting E^r from 1 to 80%. When bridged via its gelatin-binding domain to E^s-gel, 180K-opFnf promotes adherence to monocyte fibronectin receptors without phagocytosis (Czop and Austen, 1982).

The mechanism by which 180K-opFnf augments monocyte phagocytosis involves its binding to fibronectin receptors. Occupation of fibronectin receptors with particle bound intact human plasma fibronectin blocks the augmented response provided by 180K-opFnf but does not interfere with monocyte phagocytic responses to unopsonized particulate activators or to the Fc portion of IgG (Czop and Austen, 1982). Thus, 180K-opFnf opsonizes particulate activators and links them to monocyte fibronectin receptors which promotes their ingestion through monocyte β-glucan receptors. The opsonic and monocyte adherence domains within 180K-opFnf are functionally separable with monoclonal antibodies to intact human plasma fibronectin (Czop *et al.*, 1985b). When Fab fragments are incubated with 180K-opFnf before and after its opsonization of E^r, two different patterns of inhibition are observed. As shown in Fig. 6, monoclonals BD4 and CE9 inhibit the phagocytosis-enhancing activity of 180K-opFnf before and after its opsonization of E^r indicating that these monoclonals interfere with the adherence of 180K-opFnf to monocyte fibronectin receptors. Monoclonals AB3 and

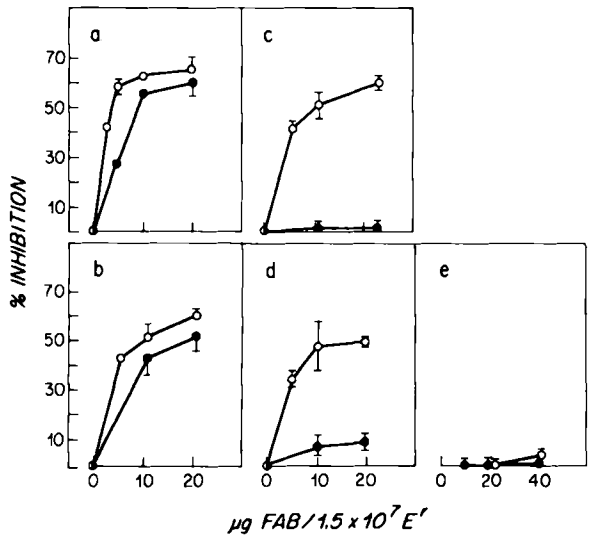


FIG. 6. Dose effects of Fab fragments of monoclonal BD4 (a), CE9 (b), AB3 (c), CPG1 (d), and BC7 (e) on the phagocytosis-enhancing activity of 500 ng of 180K-opFnf when added before (○) and after (●) its opsonization of E^r. In the absence of inhibitor, the average percentage of monocytes ingesting ≥ 1 E^r was 56% (range 51–60%). Reprinted from Czop *et al.* (1985b) with permission of The American Association of Immunologists, copyright owner.

CPG1, however, only inhibit the enhancing activity of 180K-opFnf before but not after its opsonization of E^r indicating that these monoclonals interfere with the opsonic function of 180K-opFnf. Thus, the adherence domain within 180K-opFnf is immunologically similar to the cell-adhesive region within intact human plasma fibronectin, whereas the opsonic domain is immunologically similar to regions within the carboxy-terminus of the intact molecule.

Of more physiologic interest are the findings that various isolated proteolytic cleavage fragments generated from intact fibronectin can inhibit the adherence or opsonic functions of 180K-opFnf (Czop *et al.*, 1985b). Three fractions containing cathepsin D-derivatives of the intact molecule inhibit the enhancing activity of 180K-opFnf. As summarized in Fig. 7, fragments containing the BD4 epitope block the adherence function of 180K-opFnf, whereas fragments which contain the interchain disulfide bonds and the AB3 and CPG1 epitopes block the opsonic function of 180K-opFnf. Intact fibronectin at 250-molar excess has no effect on the phagocytosis-enhancing activity of 180K-opFnf. That fragments generated from the native fibronectin molecule can compete with 180K-opFnf may be physiologically relevant to host defense.

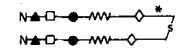
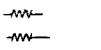
CATHEPSIN D FRACTION	BINDING PROPERTIES	MAJOR PROTEIN(S)	EPITOPE(S) PRESENT	FUNCTION OF 180K-opFnf AFFECTED	
				ADHERENCE	OPSONIC
Intact Fibronectin			AII	−	−
GEL-, HEP+ 0.1 M NaCl Monoclonal CE9-	CPGI-		BD4	+	−
	CPGI+		AB3 CPGI	−	+
GEL-, HEP+ 0.5M NaCl	CPGI-			−	−
	CPGI+		AB3 CPGI	−	+

FIG. 7. Graphic representation of the fragments of intact fibronectin used in competition experiments with 180K-opFnf that were obtained by cathepsin D cleavage and sequential fractionation on gelatin-, heparin-, and monoclonal CE9-, and/or monoclonal CPG1-Sepharose relative to their locations within a model of human plasma fibronectin. The arrangement of the functional domains within the inact molecule are symbolically shown as N-terminus (N-▲), gelatin-binding domain (□), 18,000 molecular weight link between gelatin and cell-adhesive domains (●), cell-adhesive domains (⁓), and high affinity heparin (◇). A subunit chain difference is depicted by an asterisk (*) above the α-chain and its derivatives. Reprinted from Czop *et al.* (1985b) with permission of The American Association of Immunologists, copyright owner.

VIII. Conclusions

This review has dealt with the mechanisms by which zymosan and nonmicrobial particulate activators of the alternative complement pathway are recognized, initiate phagocytic responses, and activate additional cellular responses by mononuclear phagocytes. In the absence of opsonins, phagocytic cells directly recognize these target activators as foreign and rapidly ingest them. The signals which trigger this phagocytic response are specific carbohydrate constituents and/or the conformations imposed by these constituents which interact with distinct nonimmune receptors. The phagocytic receptors for particulate activators have appropriately been designated as β-glucan receptors on the basis of the selective capacities of β-glucans with 1,3 and/or 1,6 linkages to inhibit phagocytosis of zymosan particles as well as nonmicrobial activators such as rabbit erythrocytes. The β-glucan receptors are present on human and mouse mononuclear phagocytes and appear to be as prevalent on phagocytic cells as Fc receptors for IgG. The cellular responses activatable by zymosan ingestion include the release of numerous lysosomal enzymes and *de novo* synthesis of enzymes, such as plasminogen

activator. Zymosan and the mannan-free yeast glucan particles activate oxidative metabolism of arachidonic acid, the major lipid component of membrane phospholipids. Prostaglandins, prostacyclin, and thromboxane are rapidly generated from arachidonate by cyclooxygenase enzymes and are rapidly released from the cells. Leukotrienes are also rapidly released after oxidative conversion of arachidonic acid through the 5-lipoxygenase pathway and this response is far greater by phagocytosis through β-glucan than Fc-IgG receptors.

Because the β-glucan receptor has no requirement for IgG or complement fragments, its interactions with another opsonic protein have been reviewed. This section of the review dealt with the opsonic effects of intact plasma fibronectin and of a 180,000 molecular weight fibronectin fragment, 180K-opFnf. The opsonic function of the intact molecule in physiologic responses is unclear. Soluble intact plasma fibronectin fails to bind to nonmicrobial, non-gelatin-coated targets, has little or no capacity to enhance or to induce phagocytic responses to gelatin-free microbial or nonmicrobial targets, and exhibits little specific binding to human or mouse mononuclear phagocytes in the absence of gelatin. Unlike the soluble intact molecule, 180K-opFnf contains an opsonic domain which becomes at least partially accessible within intact fibronectin when it is immobilized. Particle-bound 180K-opFnf binds to monocyte fibronectin adherence receptors and fails to trigger a phagocytic response in the absence of an independent phagocytic signal. In the absence of gelatin, 180K-opFnf only augments phagocytosis of particulate activators and does not affect Fc- or C3b-mediated functions. The opsonic domain of 180K-opFnf specifically recognizes particulate activators and is, therefore, the functional entity that limits the enhancing activity of 180K-opFnf to phagocytic responses mediated by the β-glucan receptor. This selective opsonic function distinguishes 180K-opFnf from generated opsonic C3 fragments which can deposit on virtually any surface and promote enhanced phagocytosis through Fc and β-glucan receptors.

It is highly likely that β-glucan receptors are part of an essential nonimmune clearance system. The presence of a particulate activator can rapidly initiate assembly and amplification of a host defense system involving β-glucan receptors, the cellular and humoral components of which are fully activatable in the absence of adaptive immunity.

ACKNOWLEDGMENTS

I wish to thank Donna Zepf, Cindy Stompor, Chae Choi, and Stephanie Bourdelle for their skillful technical assistance during various phases of this work. I extend special appreciation to Dr. Julian Kadish for critically reading this manuscript and for many informative discussions.

This work was supported by Grants AI-17132, AI-22531, AI-10356, and RR-05669 from the National Institutes of Health and by a grant from the New England Peabody Home Foundation.

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