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Immunology

Distinct *H-2*-linked *Ir* genes control both antibody and T cell responses to different determinants on the same antigen, myoglobin*

(major histocompatibility complex/immune response/genetics)

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ABSTRACT **The murine antibody and T lymphocyte proliferative responses to sperm whale myoglobin (Mb) were found to be under control of two distinct *H-2*-linked immune response (*Ir*) genes (*Ir-Mb-1*, mapping in the *I-A* subregion, and *Ir-Mb-2*, mapping in *I-C*). *H-2*^{d} mice (B10.D2 and DBA/2), with both genes, were high responders to Mb and its fragments for both antibody secretion and T cell proliferation, while *H-2*^{b} (B10) and *H-2*^{k} (B10.BR) mice were low responders. Strains with only *Ir-Mb-2* [B10.A and B10.A(5R)], which were intermediate responders to Mb, made antibodies to and proliferated in response to the NH_2-terminal fragment (1–55) but not the COOH-terminal fragment (132–153) when immunized with Mb. In contrast, mice carrying only the *Ir-Mb-1* gene (D2.GD and B10.GD) made antibodies to and proliferated in response to both fragments. However, their proliferation to fragment (1–55) was often lower than that of their congenic high responders (DBA/2 and B10.D2, respectively), possibly because they respond to only some of the determinants on this NH_2-terminal fragment. Thus, these data demonstrate that distinct *Ir* genes, mapping in separate *I*-subregions of *H-2*, control responses to different antigenic determinants on the same protein molecule. Moreover, the gene that controls the T lymphocyte responses to a given determinant also controls production of antibodies specific for that same determinant (or a closely associated one).**

The mechanism of action of major histocompatibility complex-linked immune response (*Ir*) genes has been a major question in immunology for a decade (for a review, see ref. 1). Results of experiments using small molecule "haptens" attached to large protein "carriers" as immunogens demonstrated that the specificity of *Ir* genes was for the carrier moiety rather than for the hapten to which antibodies were produced (2). This observation suggested expression of *Ir* genes in helper T cell function. Conversely, studies of antibody responses in congenic mice to the natural protein antigen, staphylococcal nuclease, demonstrated that *Ir* gene(s) controlled the relative proportion of antibodies made to two distinct regions of the same molecule (3). Because these congenic mice, which differ only at *H-2*, share the same immunoglobulin structural gene repertoire, *Ir* gene(s) for nuclease appeared to regulate the selection of B cell clones in antibody synthesis. However, the same gene(s) appeared to control both the antibody response to the same fragments of nuclease used as immunogen (4) and the T cell proliferative response to nuclease and its fragments (5). Thus, the question arose whether a single gene was involved in selection of both B cell and T cell clones to a given antigenic determinant of nuclease. The answer to this question, with respect to nuclease, has been elusive.

Responses to the synthetic antigen, $Glu^{53}Lys^{36}Phe^{11}$, appear to be under the control of two complementing *Ir* genes, both of which must be present to obtain a response (6). Recently, we have shown the antibody response to another natural protein antigen, sperm whale myoglobin (Mb), to be under the control of at least two *H-2*-linked *Ir* genes, which map in distinct subregions of the *I* region of *H-2* (7). In contrast to the experience with $Glu^{53}Lys^{36}Phe^{11}$, each of these genes alone appears to be sufficient to produce at least partial responsiveness to the whole Mb molecule. We investigated the possibility, therefore, that each of the two *Ir* genes may control independently the response to a different determinant on the same Mb molecule (8). Preliminary studies have suggested this to be the case (8, 9).

We now show that genes mapping in different *I* subregions, and which are therefore genetically distinct, independently regulate responses to chemically discrete regions of the same protein antigen. Furthermore, these determinant-specific genes appear to control both the synthesis of B cell products (antibodies) and T cell proliferative responses to their respective determinants. This result suggests the possible existence of determinant-specific helper T cells specific for the same determinant to which antibodies are made. It also suggests a common control mechanism for antigen-stimulated T cell proliferation and antigen-induced stimulation of B cell antibody synthesis, perhaps through helper T cell recognition of antigen in association with the same *I* region associated (Ia) antigenic determinants on macrophages and B cells.

MATERIALS AND METHODS

Animals. C57BL/10Sn (B10), B10.A/SgSn, B10.A(5R)/SgSn, B10.BR/SgSn, B10.D2/nSn, and DBA/2J mice were obtained from Jackson Laboratory. B10.A/Sx, B10.S/Sx, and D2.GD/Sx mice were the gift of David H. Sachs; B10.GD mice were the gift of Martin E. Dorf and Baruj Benacerraf. Mice were 8–22 weeks old and were of either sex.

Antigens. Mb was obtained from the Accurate Chemical and Scientific Co. (Hicksville, NY). The major chromatographic component, IV, purified as described (7), was used throughout these studies. Fragments of Mb corresponding to amino acid residues 1–55 and 132–153 were prepared by cyanogen bromide cleavage and gel filtration (10). Amino acid analysis of acid hydrolysates of the peptides excluded contamination with other peptides of Mb any greater than 0.5% (limits of detectability).

Labeling of Fragments and Radiobinding Assay. Similar to the labeling of whole Mb (7), each fragment was labeled with 0.2 M $K^{14}CNO$ (60 Ci/mol, Amersham/Searle; 1 Ci = 3.7×10^{10} becquerels) by the method of Stark (11) at pH 7.0 for 60–90 min at 25°C to label preferentially the NH_2-terminal α-amino group. The number of moles of ^{14}CNO incorporated per mole of fragment was 1.12 for fragment (1–55) and 1.15 for fragment (132–153).

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Abbreviations: *Ir*, immune response; Mb, sperm whale myoglobin; *Ir-Mb-1*, *Ir* gene controlling responses to Mb in *I-A* of *H-2*; *Ir-Mb-2*, *Ir* gene controlling responses to Mb in *I-C* of *H-2*.

* This paper is no. 2 in a series. Paper no. 1 is ref. 7.

Immunization of mice for antibody studies and binding of ^{14}C-labeled Mb and fragment (132–153) by antisera in a polyethylene glycol precipitation assay were performed as described (7). Complete binding curves were determined to assess the total concentration of antibody capable of binding the antigen at large free antigen excess.

Antigen-Stimulated T Cell Proliferation. Mb-primed lymph node cells were prepared by the method of Corradin *et al.* (12). Animals were injected subcutaneously at the base of the tail with antigen emulsified in complete Freund's adjuvant (H37Ra, Difco). Eight days later, cells from the draining lymph nodes were cultured at a concentration of 2×10^6/ml in 0.2 ml of RPMI 1640 containing 2 mM glutamine, 25 mM Hepes, 100 units penicillin and 100 μg of streptomycin per ml (GIBCO), 50 μM 2-mercaptoethanol (Microbiological Associates, Bethesda, MD), and 10% fetal calf serum (Reheis Chemical Co., Chicago, IL) with or without the addition of increasing amounts of Mb, fragment (1–55), or fragment (132–153). In some experiments, the cells were enriched for the presence of T cells by passage through nylon wool (13). After 5 days, 0.5 μCi of [^3H]methylthymidine (New England Nuclear, specific activity 6.7 Ci/mmol) was added and, after 4 additional hours at 37°C, cells were harvested in an automated collecting device (Microbiological Associates). Proliferation was estimated by scintillation counting of ^3H incorporated into DNA.

RESULTS

In order to assess the *Ir* gene control of the antibody response to Mb, we measured the concentration of antibodies binding specifically to radiolabeled Mb and its fragments in sera from mice immunized three times with whole Mb. As demonstrated (7), *H-2*^{d} mice (B10.D2 and DBA/2) produced high levels of antibody, as assessed from the plateau of the binding curves at antigen excess (Figs. 1*A* and 2*A*, respectively). *H-2*^{b} and *H-2*^{k} mice (B10 and B10.BR) produced low levels. The *H-2* linkage shown by variation in antibody levels among congenic strains, which differ only at *H-2*, was confirmed by a formal analysis of the progeny of a backcross of (B10 × B10.D2) × B10 (unpublished observations). Two genes controlling this response have been mapped in the *I-A* and *I-C* subregions of the *I* region of the *H-2* complex (7). For ease in following the genetics, the haplotype origin of the *I-A* and *I-C* subregions of each strain will be shown in parentheses. The recombinant congenic strains B10.A(5R) and B10.A, bearing the high responder *d* haplotype in the *I-C* subregion but the low responder *b* or *k* haplotypes in the *I-A* subregion [(*b,d*) and (*k,d*), respectively], produced intermediate levels of antimyoglobin (Fig. 1*A*). Both levels were lower than that of strain B10.D2 (*d,d*) but the level in the B10.A(5R) (*b,d*) sera was 4 times that of the corresponding low responder B10 (*b,b*) and the level in the B10.A (*k,d*) sera was 6 times greater than that in the corresponding low responder B10.BR (*k,k*).

About 10% of the antimyoglobin antibodies in the B10.D2 (*d,d*) sera reacted with the labeled COOH-terminal fragment (132–153), whereas the B10.BR (*k,k*) sera showed no significant reaction (Fig. 1*B*). In contrast to the binding of whole Mb, the B10.A (*k,d*) antimyoglobin sera did not contain more antibodies to this region of Mb than did the B10.BR (*k,k*) sera. Similarly, although the low responder B10 (*b,b*) antimyoglobin contained some antibodies to region (132–153), the B10.A(5R) (*b,d*) antimyoglobin did not contain any more than did the B10 (*b,b*). Thus, the *d* haplotype in the *I-C* subregion allows antibody production to some part of Mb, but is not sufficient for production of antibodies to the COOH-terminal region (residues 132–153).

In contrast, the D2.GD (*d,b*) strain, with the *d* haplotype in *I-A* but the low responder *b* haplotype in *I-C*, made just as much antimyoglobin as the congenic strain, DBA/2 (*d,d*) (Fig. 2*A*). Moreover, the antimyoglobin sera of these two strains contained equivalent amounts of antibody binding to the labeled fragment (132–153) (Fig. 1*B*). Thus, the *d* haplotype in *I-A* is sufficient by itself to allow production of antibodies to this region.

We conclude that the *Ir* gene in *I-A*, denoted *Ir-Mb-1*, controls the antibody response to a determinant in the region from residues 132 to 153, as well as the responses to other regions, because antibodies to this COOH-terminal region constitute only about 10% of the total antimyoglobin. In contrast, the gene in *I-C*, denoted *Ir-Mb-2*, controls responses to determinant(s) in parts of the molecule other than this one. We are unable to define the determinants for which the antibody response is regulated by the *Ir-Mb-2* gene. Because of nonspecific precipitation, we have been unable to obtain satisfactory quantitative measurements of the antibody binding capacity to these

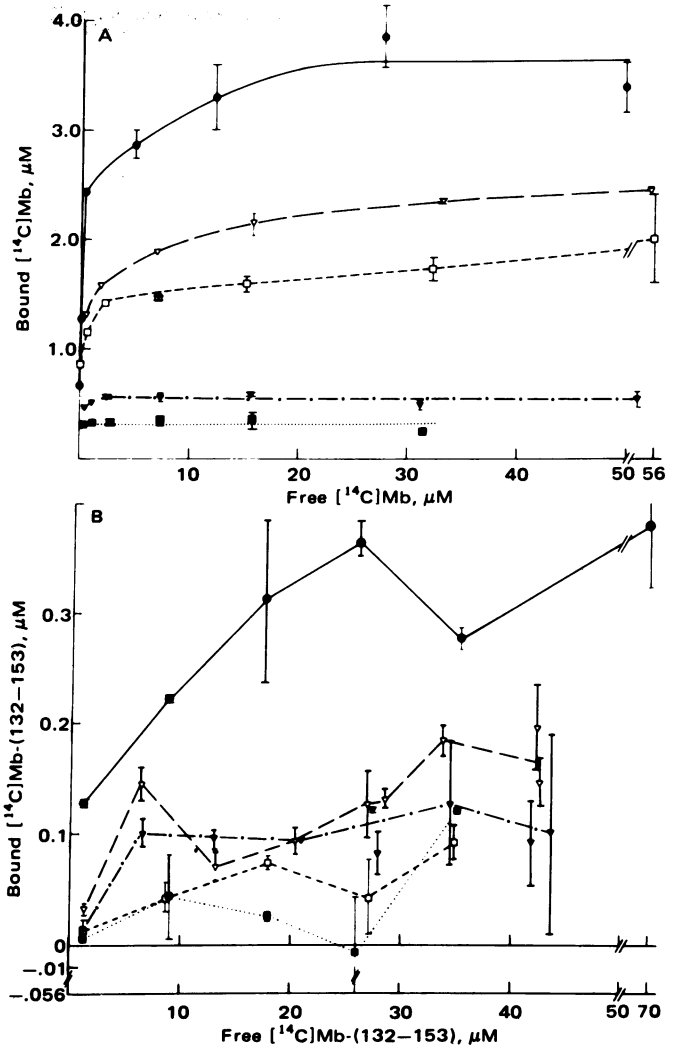

FIG. 1. Binding curves for antibodies to Mb reacting with ^{14}C-labeled Mb or its fragment (132–153). The concentration of antibodies specific for the given antigen is determined from the plateau multiplied by the dilution factor used for all sera (5×). (*A*) Whole Mb; (*B*) the same sera binding to fragment (132–153). Sera were from seven B10.D2 mice (—●—); six B10.A mice (– –□– –); eight B10.A(5R) mice (—▽—); five B10.BR mice (.....■.....); and nine B10 mice (–·– ▼ –·–). All strains used are congenic (i.e., differ only at *H-2*).

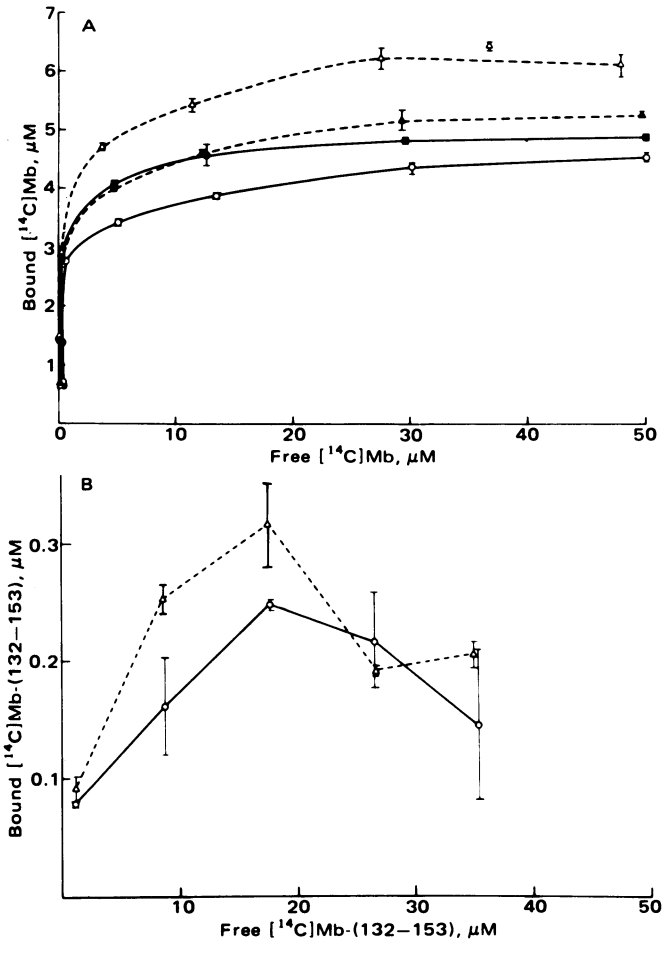

FIG. 2. Binding curves for antibodies to Mb reacting with ^{14}C-labeled Mb or its fragment (132–153). (*A*) Whole Mb; (*B*) the same sera binding to fragment (132–153). Sera were from eight DBA/2 mice (—o—); and eight D2.GD mice (- - - -Δ- - - -). Strains are congenic. (*A*) DBA/2: o, tertiary bleed; ●, secondary bleed. D2.GD: Δ, tertiary bleed; ▲, secondary bleed.

fragments. We could, however, demonstrate that the antimyoglobin sera of recombinants B10.A (*k,d*) and D2.GD (*d,b*) both bound ^{14}C-labeled fragment (1–55) in excess of the pre-immune sera (data not shown). Therefore, it appeared that either gene alone could allow a response to some determinants in this NH_2-terminal third of the Mb molecule.

In order to assess responses to both the COOH-terminal and NH_2-terminal fragments by the same method, as well as to compare the genetic regulation of the determinant-specific antibody and T cell immune responses, we tested the antigen-stimulated proliferative responses of T cells from the draining nodes of mice immunized with whole Mb. The results exactly paralleled the magnitude and specificity of the antibody responses. Initial experiments demonstrated that T cells from B10.D2 mice, immunized *in vivo* with 10, 20, 50, or 100 μg of Mb in adjuvant, proliferated well when stimulated *in vitro* with Mb. T cells from B10 mice immunized *in vivo* with 10 or 20 μg of Mb failed to respond, whereas those from mice immunized with 50 or 100 μg produced a small but definite response (data not shown). Therefore, in order not to override any *Ir* gene control at high doses of immunogen, subsequent experiments used an *in vivo* immunization of 10–20 μg of Mb in complete Freund's adjuvant. To demonstrate that the proliferation measured was a function of the T cells cultured, we performed some experiments with lymph node cells that had been passed through nylon wool (13). These cell populations contained less than 5% phagocytic and 2% surface immunoglobulin-positive cells (data not shown). Results of these experiments exactly paralleled those performed with unseparated lymph node cell populations. In addition, pretreatment of lymph node cells with anti-Thy 1.2 and complement abrogated the response.

The proliferative responses to Mb of lymph node T cells from Mb-immunized B10.D2 (*d,d*) mice were high, those from B10 (*b,b*) and B10.BR (*k,k*) were low, and those from the intermediate antibody responders B10.A (*k,d*) and B10.A(5R) (*b,d*) were intermediate (Fig. 3*A*). T cells from intermediate responders, which proliferated when stimulated with whole Mb, failed to proliferate in response to fragment (132–153) (Fig. 3*C*), in analogy with the antibody results. However, they did proliferate in response to fragment (1–55), sometimes almost as well as to whole Mb (Fig. 3*B*). Thus, the *d* haplotype of the *Ir-Mb-2* gene is sufficient to allow a T cell response to the NH_2-terminal region but not to the COOH-terminal region of Mb.

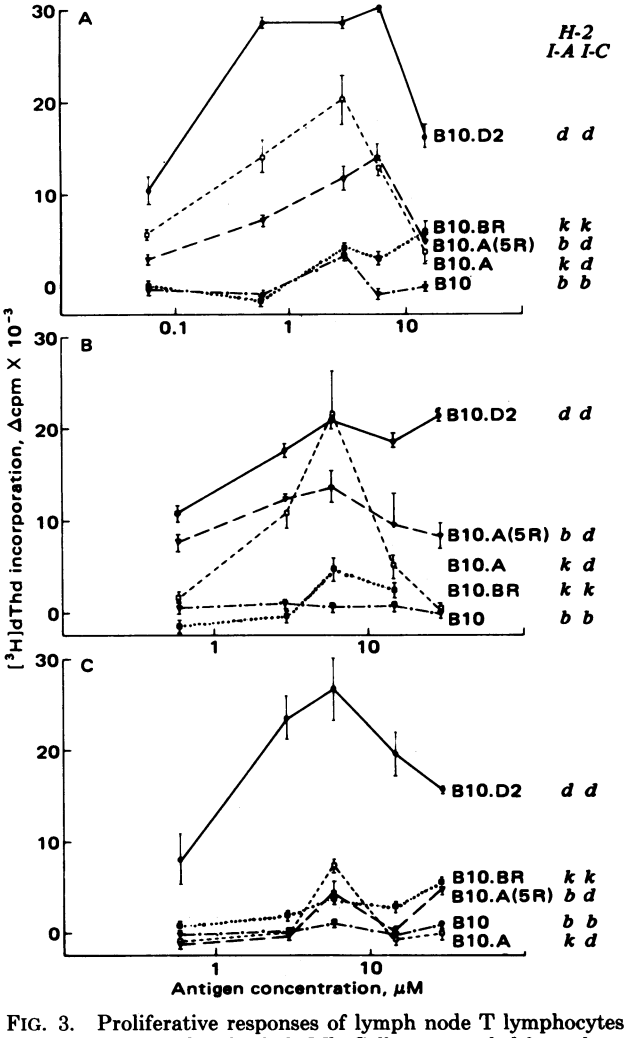

FIG. 3. Proliferative responses of lymph node T lymphocytes from mice immunized with whole Mb. Cells were pooled from three to four mice and cultured with the final concentrations of antigens shown. (*A*) Mb; (*B*) fragment (1–55); (*C*) fragment (132–153). The same cell populations were tested simultaneously for responses to each of the three antigens. Points represent geometric mean $\times/+$ SEM of [^{3}H]dThd incorporation of triplicate cultures, less the mean incorporation of medium controls, usually 3–9000 cpm. Several repetitions of the experiments with other mice yielded similar patterns of response. Symbols for congenic strains as in legend to Fig. 1.

In contrast, T cells from Mb-immunized B10.GD (*d,b*) mice, with only *Ir-Mb-1*, proliferated in response to whole Mb and to the COOH-terminal fragment (132–153) at least as well as the congenic B10.D2 (*d,d*) mice with both genes (Fig. 4 *A* and *C*). Thus, the *Ir-Mb-1* gene is sufficient to produce a response to this region of Mb. However, T cells from the B10.GD (*d,b*) mice were stimulated by the NH_2-terminal fragment (1–55) less than were those from the B10.D2 (*d,d*) mice (Fig. 4*B*). In other experiments with these same strains, or in comparisons of D2.GD (*d,b*) and congenic DBA/2 (*d,d*) mice, the same patterns were seen, although sometimes the responses to fragment (1–55) were as high in the D2.GD strain as in the DBA/2 strain (data not shown). We conclude that the *Ir-Mb-1* gene allows a response to some determinant(s) in the region between residues 1 and 55, although perhaps not to all the determinants in this region to which *H-2*^{d} mice (with both genes) respond. We do not know whether multiple determinants in this region are immunogenic in *H-2*^{d} mice. It is possible that the determinant in this region that stimulates mice with only *Ir-Mb-1* may be different from the one that stimulates mice with only *Ir-Mb-2*.

It should be emphasized that in all cases we are studying responses after immunization with native Mb. The fragments, which undergo denaturation during CNBr cleavage, are used merely as *in vitro* probes of these responses. This approach is feasible for the antibody studies because the fragments are in equilibrium with the native conformation and for the proliferation studies because the native conformation may not be necessary for such T cell responses (5).

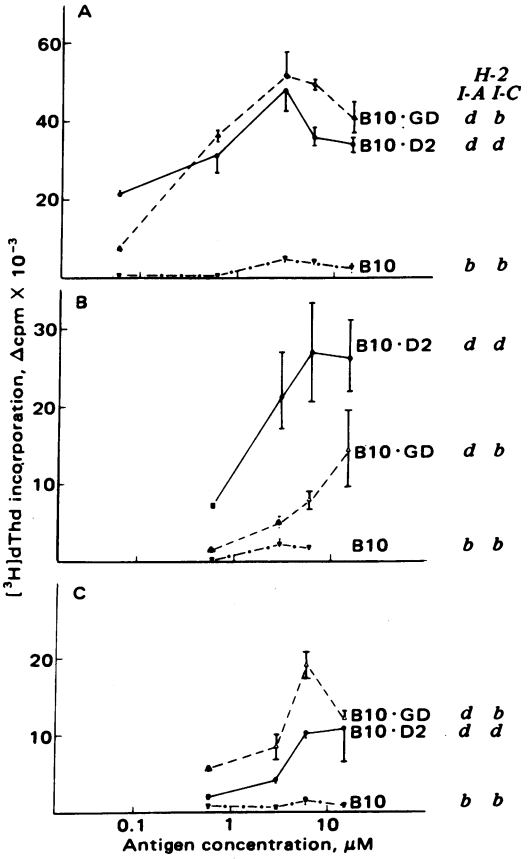

FIG. 4. Proliferative responses of lymph node T lymphocytes from mice immunized with Mb. See legend to Fig. 3. (*A*) Whole Mb; (*B*) fragment (1–55); (*C*) fragment (132–153).

DISCUSSION

The results presented demonstrate that the two myoglobin *Ir* genes in the *H-2*^{d} haplotype, *Ir-Mb-1* mapping in *I-A* and *Ir-Mb-2* mapping tentatively in *I-C* (to the right of *I-E* and the left of *H2-D*) (7), individually control responses to different determinants on the same antigen molecule. Recombinant congenic strains that have only *Ir-Mb-2*, such as B10.A and B10.A(5R), respond to determinants of whole Mb and the NH_2-terminal region, from residues 1 to 55, but not to any determinants in the COOH-terminal region, from residues 132 to 153. These findings explain their intermediate level responsiveness to whole Mb in both T cell proliferation and antibody response. Their lower proliferative responses even to fragment (1–55) suggest that these strains with only *Ir-Mb-2* may recognize only a subset of the determinants in this region that are recognized by B10.D2. Although Atassi and his coworkers (14) found only one determinant in the NH_2-terminal region that was recognized by antibodies raised in goats and rabbits, it is entirely possible that more than one determinant in this 55-residue sequence is immunogenic in the mouse.

In contrast, when immunized with whole Mb, recombinant congenic strains with only *Ir-Mb-1*, such as B10.GD and D2.GD, respond at least as well to the COOH-terminal region of Mb as do their congenic *H-2*^{d} counterparts (B10.D2 and DBA/2, respectively) in terms of both antibody production and T cell proliferation. Thus, *Ir-Mb-1* alone is sufficient for responses to a determinant between residues 132 and 153, while *Ir-Mb-2* is not. This reciprocal pattern of control confirms our hypothesis that these different genes control responses to different antigenic determinants. If the gene in *I-C* does not require complementation with one in *I-A*, this would be the first example of such an independent *Ir* gene in *I-C*. However, it remains formally possible that the high responder *H-2*^{d} allele in *I-C* can complement with any haplotype in *I-A*, including the low responder *k* and *b* alleles. Thus, we cannot exclude the possibility that other genes within or outside of *H-2* are necessary to complement with these two genes to regulate the responses described. Although we have already shown the existence of a non-*H-2*-linked gene(s) which, superimposed on the *H-2*-linked control, regulates the magnitude of the antibody response to myoglobin (7), the existence of these other genes does not contradict the conclusion that different genes within *H-2* control responses to different antigenic determinants on Mb.

Antigenic determinants or regions for which the antibody or T cell response is under *H-2*-linked *Ir* gene control have been defined for other natural protein antigens, including staphylococcal nuclease (3, 5, 15), insulin (16, 17), cytochrome *c* (18, 19), and lysozyme (20). Also, Mozes *et al.* (21) showed that two murine genes were involved in antibody responses to the synthetic polypeptide antigen, poly(Phe,Glu)-poly(Pro)--poly(L-Lys). One of these, in *H-2*, affected anti-(Phe,Glu) copolymer antibody responses, while the other, a non-*H-2*-linked gene, affected those to the Pro-Lys "backbone." However, the present report demonstrates that genetically distinct *H-2*-linked *Ir* genes, mapping in different *I* subregions, independently control antibody and T cell proliferative responses to chemically distinct antigenic determinants on the same natural globular protein.

This phenomenon may, in retrospect, explain several observations reported previously. The existence of intermediate responder mouse strains to lactate dehydrogenase (LDH_B), which complement in the F_1 hybrid to produce high responders (22), may be due to genes in each of the intermediate strains which allow responses to some of the determinants on LDH_B but not to others. The F_1 hybrid, responding to both sets of

determinants recognized by the parental strains, would therefore demonstrate higher responses than those seen in either parent. Thus, some types of complementation may arise by this mechanism.

A logical extension of the present results is that each antigenic determinant on a complex multideterminant antigen is under the control of a distinct *Ir* gene.† The detection and mapping of an *Ir* gene requires the existence of a low responder strain. Thus, the reason that complex antigens, such as keyhole limpet hemocyanin, appear not to be under *Ir* gene control may be that all strains respond to enough determinants to be classified as high responders. However, one might theoretically be able to demonstrate a strain that is a low responder to any particular determinant. This line of thought may be extended to suggest that one reason that most *Ir* genes are defined by high and low responders rather than nonresponders is that even low responders respond to some determinants. This idea is corroborated by the observation that, in guinea pigs, all-or-none responsiveness occurs with the simple antigen poly(L-lysine) (1), which may consist of but a single determinant.

Finally, we must consider the implications for the mechanism of *Ir* gene control. The gene that controls production of antibodies to a given region of the antigen molecule also controls T cell proliferation to that region, even though the sensitivities of T and B cells to conformational changes may be different (4, 24). This finding suggests a symmetry in *Ir* gene control of T and B lymphocyte responses, which has been proposed by others to explain certain genetic restriction phenomena (25). The idea is that the T cell, in order to be triggered, must recognize a particular combination of immunizing antigen and *Ir* gene product (Ia antigen) on the surface of the antigen-presenting cell (usually thought to be a macrophage). Then, to help trigger a B cell, this T cell must recognize the same combination of antigenic determinants and Ia determinants on the B cell. If no clone of T cells exists that recognizes the available combinations of Ia antigen and antigenic determinant, an *Ir* gene defect exists for both T cell and B cell responses.

While this hypothesis has yet to be proven, several recent results with the Mb system support it. First, it would appear that the T cells that are proliferating include a population of T helper cells. Preliminary data suggest that they bear the Ly phenotype of helper cells, Ly 1^+2^- (unpublished observations), and that they can subserve helper function *in vitro* after blastogenesis (J. Chiller, personal communication). Second, we have demonstrated that the proliferative response of macrophage-depleted, Mb-primed T cells from an F_1 hybrid between a strain carrying both *Ir-Mb* genes and one carrying neither is determined completely by the *Ir* phenotype of the donor of macrophages used to reconstitute the system *in vitro* (ref. 26 and L. K. Richman, W. Strober, and J. A. Berzofsky, unpublished data). Thus, the function of both *Ir* genes is determined by the macrophage, which, in turn, selects those determinants that stimulate proliferation *in vitro* (and, presumably, *in vivo*). This phenomenon serves to elucidate the "determinant selection" mediated by macrophages which was described by Rosenthal (27) for the proliferative response to insulin.

† If, as some theories suggest (23), the number of *Ir* genes is limited by the number of Ia antigen specificities, then a single Ia antigen may determine the *Ir* gene control of more than one determinant on a complex antigen. However, the point would still hold that the response to a multideterminant antigen would be controlled by many *Ir* genes.

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