IR GENE REGULATION OF THE RESPONSE TO TRINITROPHENYL-POLYSACCHARIDES

Two Independent Genes are Required for Antibody Production*

BY LINDA M. HILLSTROM AND JOHN E. NIEDERHUBER

From the Department of Microbiology and Immunology and the Department of Surgery, The University of Michigan Medical School, Ann Arbor, Michigan 48109

Regulation of the immune response by genes located in the major histocompatibility complex (MHC)[1] has been extensively investigated (1–5). As a result of these studies, it has been concluded that the *Ir* genes of the MHC code for cell surface structures termed Ia antigens. Recognition of appropriate Ia determinants on antigen-presenting cells by Ly-1$^+$ T cells is required for successful induction of the response (6–9). While the mechanisms of *Ir* gene control are poorly understood, it is clear that regulation at the level of the macrophage and at the level of the appropriate T cell are both critical (10–11).

Current understanding of immune response regulation has been derived largely from studies using T-dependent antigens. Indeed, it could be postulated that T-independent (TI) antigens would not demonstrate the same degree of specificity or *Ir* gene regulation. Recent evidence, however, suggests that even traditional TI antigens require at least some T cells (12–13) to produce an optimum response.

In an effort to investigate the role of *Ir* genes and their products in the response to TI antigens, we have analyzed the genetic restriction of the response to the TI-2 antigen 2,4,6-trinitrophenyl (TNP)-Ficoll. TNP-Ficoll, a prototypic TI-2 antigen, requires Ia$^+$ macrophages (14), a specific subset of B cells (Lyb-5$^+$) (15), and elicits a response in congenitally athymic mice (16). The response of nu/nu mice to TI-2 antigens suggested that T cells were not required for specific antibody production. However, rigorous depletion of T cells from spleen cell cultures abrogated the response to TNP-Ficoll which was in turn restored by the addition of small numbers of purified T cells (12–13). These observations indicated that the response to TNP-Ficoll required T cells, although not to the extent necessary to generate a response to a T-dependent antigen.

We have extended these studies to demonstrate that the in vitro antibody response to TNP-Ficoll is under strict *Ir* gene control. This control mapped to two complementing loci, one located in or to the left of the *I-A* subregion and another to the right of the *I-E* subregion. In addition, the response to two structurally different polysaccharides, TNP-dextran and TNP-mannan, followed the same pattern of restriction. Finally, experiments demonstrated blocking of macrophage presentation of TNP-

* Supported in part by grant CA-25052-04 AI from the National Institutes of Health.

[1] *Abbreviations used in this paper:* AECM, aminoethyl carbamethyl; FCS, fetal calf serum; GLϕ, the random linear synthetic terpolymer of poly-(L-glu, L-lys, L-phe); MHC, major histocompatibility complex; PFC, plaque-forming cell; TI, T independent; TNP, trinitrophenyl.

J. EXP. MED. © The Rockefeller University Press · 0022-1007/83/06/2002/15 $1.00
Volume 157 June 1983 2002–2016

Ficoll by treatment with appropriate antiserum directed against either the *I-A* subregion of the responder haplotype or against responder alleles mapping in or to the right of *I-E*.

Materials and Methods

Mice. All mice were bred in our colony at the University of Michigan and used when 2–6 mo of age. Original breeders were obtained from The Jackson Laboratory, Bar Harbor, ME or from Dr. Donald Shreffler, Washington University, St. Louis, MO.

Antigens. TNP-aminoethyl carbamylmethyl (AECM)-Ficoll was obtained from Biosearch Laboratories, San Rafael, CA. Dextran B512 (mol wt 500,000) and mannan were purchased from Sigma Chemical Co., St. Louis, MO. The AECM derivative of each was prepared using the method described by Inman (17). The TNP conjugate of each was prepared according to published procedures (18). The conjugation ratios were TNP_{34}-AECM-Ficoll, TNP_{19}-AECM-dextran. TNP-AECM-mannan contained 1.75×10^{-2} mol TNP/mg mannan.

Antisera. Restricted anti-Ia alloantisera were prepared as previously described (19–21). Batches of serum from several bleedings were tested in a dye exclusion microcytotoxicity assay for appropriate anti-Ia reactivity and antibody titer (21). The hybridoma cell line MKD6 (anti-I-A^d) was the gift of Dr. Phillippa Marrack, National Jewish Hospital, Denver, CO. Cell lines producing monoclonal antibodies directed against specificity Ia.7 (14-4.4) and Ia.17 (10-2.16) were obtained from the Salk Cell Distribution Center, La Jolla, CA. Batches of anti-Thy-1.2 sera were prepared by immunizing A.AKR ($H\text{-}2^{al}$, $Thy\text{-}1^a$) mice with C3H ($H\text{-}2^{al}$, $Thy\text{-}1^b$) thymocytes. Rabbit anti-mouse IgG serum was prepared by injecting rabbits with rabbit erythrocytes coated with mouse anti-rabbit erythrocyte hyperimmune antibodies.

Complement for antisera testing and experiments was obtained by cardiac puncture from 2 to 4-wk-old rabbits. The complement was screened against thymocytes and lymph node cells for natural cytotoxicity and only batches with $<5\%$ natural cytotoxicity were used.

Cell Culture Conditions. Single-cell suspensions of individual spleens were prepared and seeded at 4×10^6 lymphocytes per well (four wells per condition) in Mishell-Dutton culture medium (22) with 10% Reheis fetal calf serum (FCS) (Reheis Chemical Co., Phoenix, AZ). Cultures were established for Costar 24-well plates (Costar, Data Packaging, Cambridge, MA). Antigen was added at a final concentration of 0.1 μg/ml TNP-Ficoll in a final volume of 2 ml per well. Plates were incubated for 4 d at 37°C, 7% CO_2.

In experiments involving cell separations, cells were added at the concentrations indicated. Purified cell populations were obtained using previously published procedures (23, 24). Briefly, spleen cells were allowed to adhere to plastic petri dishes (Falcon Labware, Oxnard, CA) for 2.5 h. Nonadherent cells were decanted, the adherent cells washed with Mishell-Dutton medium with 10% FCS, and treated with appropriate dilutions of anti-Thy-1.2 serum, rabbit anti-mouse IgG serum, and complement. Surviving cells were treated with mitomycin C, washed, and treated with blocking antisera for 30 min. The anti-Ia-blocked purified macrophages were harvested with cold EDTA and gentle scraping with a rubber policeman.

Macrophage-depleted spleen cells were prepared by repeated incubation of cells with carbonyl iron powder (Atomergic Chemetals Corp., Plainview, NY) and removal of phagocytic cells with a strong magnet. Cells incorporating latex particles and having morphologic characteristics of macrophages comprised $<1\%$ of this remaining cell population. Total cell loss with this procedure was 40–50%, but no difference in the T cell to B cell ratio could be discerned.

Local Hemolysis in Gel. TNP-specific IgM antibody-producing cells were assayed on day four of the culture period by a modified Jerne technique (25, 26). Sheep erythrocytes were lightly conjugated with TNP and used to determine the number of direct plaque-forming cells (PFC) (27). $>90\%$ of PFC were inhibited by 10^{-2} M TNP-lysine. Results were corrected for background PFC using unhaptenated sheep erythrocytes (28).

Statistical Analysis. Data was analyzed with the MIDAS program of the Michigan Terminal System with the assistance of the Statistical Research Laboratory, The University of Michigan.

Results

Studies of the primary immune response to TNP-Ficoll demonstrated a haplotype-dependent variation of the in vitro antibody response. To investigate this variation, five congenic strains of mice were challenged with optimum doses of TNP-Ficoll. The results of these experiments are shown in Table I. Whole spleen cultures of each strain were incubated with 0.1 μg/ml TNP-Ficoll and assayed for TNP-specific IgM-PFC on day four. The range of responses between haplotypes was quite large, and the relative differences were consistent throughout all experiments. B10.S($H\text{-}2^s$) mice gave very high responses (8,405 ± 1,303 PFC/culture) when compared with other strains tested. B10.D2($H\text{-}2^d$; 2,666 ± 942 PFC/culture), B10.A($H\text{-}2^a$; 2,285 ± 624 PFC/culture), and B10.BR($H\text{-}2^k$; 2,129 ± 988 PFC/culture) responses were higher than the B10($H\text{-}2^b$; 1,276 ± 560 PFC/culture) response, but were significantly lower than the B10.S response. Because the strains tested have identical genetic backgrounds and differ only in their *H-2* regions, the different levels of TNP response could be attributed to a gene or genes located in the *H-2* complex.

Mapping of Control. To further investigate the *I* region control of this response, intra-*I* region recombinant mouse strains were tested for their response to TNP-Ficoll. Recombinants were selected to localize the control to specific *I* subregions. The results of these experiments are shown in Fig. 1. At least seven mice of each haplotype were individually tested to ascertain the statistical significance of differences observed. As noted, $H\text{-}2^{g2}$ mice always gave the highest response and were therefore included in each experiment as a control. To statistically compare results of individual experiments, results are expressed as percent of high responder D2.GD mice normalized to 1,000 PFC/culture. The mean PFC/culture response of 23 D2.GD mice tested individually was 4,541 ± 1,046 PFC/culture. Comparison of the various congenic recombinant strains indicated that two loci were involved, one in or to the left of the *I-A* subregion and the other in or to the right of the *I-E* subregion.

Determination of the left hand locus of control was based on responses of recombinant strains whose crossover position was between the *I-A* and *I-B* subregions. Challenge of the B10 strain (*bbbbb*) resulted in a low response averaging 170 ± 33 PFC/culture, whereas strain B10.A(4R) (*kbbbb*) produced a significantly higher response of 503 ± 90 PFC/culture. Since these strains differed only in or to the left of the *I-A* subregion, their responses suggested an *H-2K* or *I-A* subregion contribution to the response. The role of an *H-2K* or *I-A* subregion product in the TNP-Ficoll response was further demonstrated by comparing B10.GD (845 ± 110 PFC/culture) mice with either of its parental haplotypes $H\text{-}2^d$ (*ddddd*; 247 ± 45 PFC/culture) or $H\text{-}2^b$ (*bbbbb*;

Table I

H-2 Control of the In Vitro Antibody Response to TNP-Ficoll

Strain	*H-2 I* region	Number of mice tested	Mean IgM PFC/culture ± SEM
B10.S	sssss	5	8,405 ± 1,303
B10.D2	ddddd	5	2,666 ± 942
B10.A	kkkkk	5	2,285 ± 624
B10.BR	kkkkk	5	2,129 ± 988
B10	bbbbb	5	1,276 ± 560

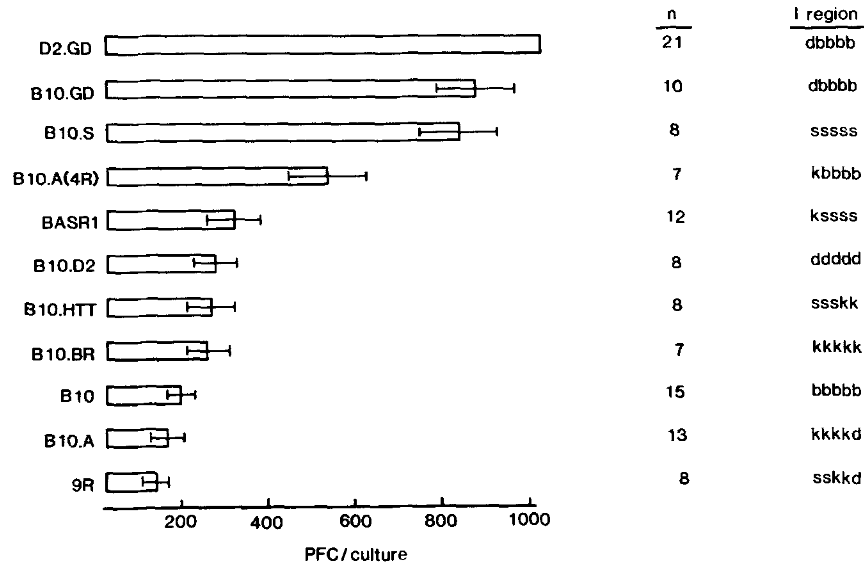

FIG. 1. Strain distribution of the primary in vitro antibody response to TNP-Ficoll. Quadruplicate wells of 4×10^6 normal spleen cells from individual mice were challenged with 0.1 μg/ml TNP-Ficoll and assayed on day four for TNP-specific IgM-PFC. Data for each experiment was normalized to a high responder (D2.GD) response of 1,000 PFC/culture of four pooled wells. Each bar represents the mean ± SEM of responses of indicated numbers of individual mice.

170 ± 33 PFC/culture). A third pair of congenic mice B10.BASR1 (*kssss*) and B10.S (*sssss*) confirmed our observations with responses of 290 ± 60 and 810 ± 95 PFC/culture, respectively. These results clearly mapped the control of the antibody response in or to the left of the *I-A* subregion, with *d* and *s* as high responder alleles. Responses of the B10.A(4R) and B10.BASR1 recombinants demonstrated that the presence of the k allele at this locus produced an intermediate response.

Responses of two additional recombinant strains implicated a second area of control located in or to the right of the *I-E* subregion. Comparison of responses of B10.S (810 ± 95 PFC/culture) and B10.S(9R) (*sskkd*; 115 ± 30 PFC/culture) indicated that a recombination to the right of the *I-B* subregion significantly reduced the ability to respond to TNP-Ficoll. The response of the B10.HTT (*ssskk*; 236 ± 51 PFC/culture) further localized the right hand region of control in or to the right of the *I-E* subregion.

To ensure that this observed variation in response was not either a dose dependent or a kinetic phenomenon, high responder and low responder spleen cells were challenged with increasing doses of the antigen TNP-Ficoll (Fig. 2) or assayed over a period of 6 d (Fig. 3). The dose response curves shown in Fig. 2 clearly illustrate that the high responder haplotype produces significantly more PFC per culture over a large range of antigen doses than the low responder haplotype under identical conditions. Similarly, Fig. 3 demonstrates that the high responder and low responder phenotypes are expressed throughout the culture period.

Investigation of Complementing Genes. To confirm the evidence that two separate, complementing loci control the response to TNP-Ficoll, F_1 hybrids were bred and tested for their ability to respond to the antigen. Strains were chosen either to contain the known high responder recombinant haplotypes or to test combinations of complementing alleles that were not available to us as recombinant strains. The results of

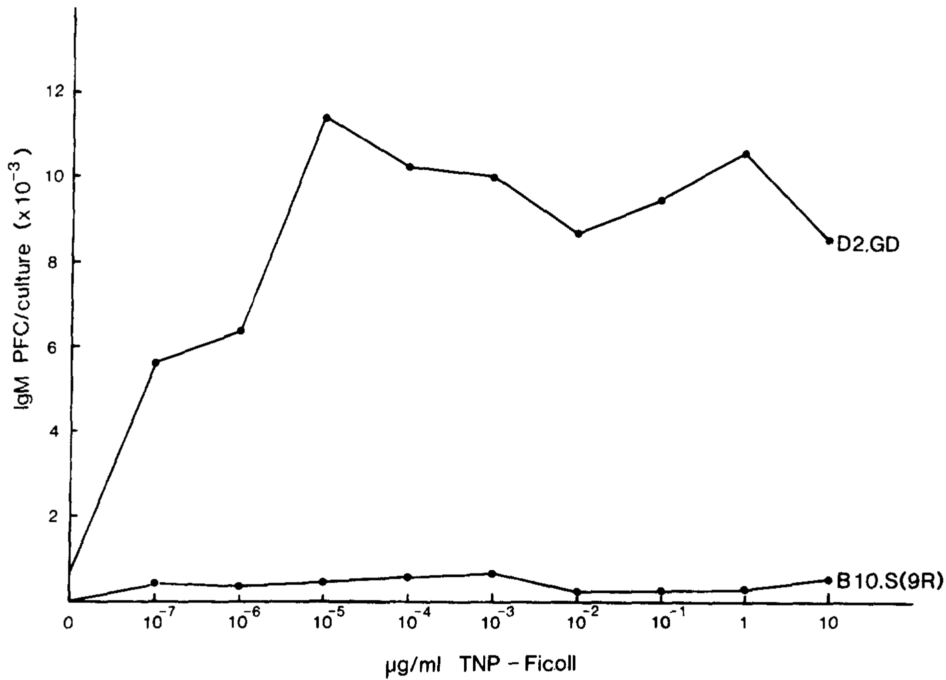

FIG. 2. The in vitro IgM-PFC response of high responder (D2.GD) or low responder [B10.S(9R)] spleen cells to increasing doses of TNP-Ficoll. Quadruplicate wells of 4×10^6 normal spleen cells from each strain were challenged with indicated doses of TNP-Ficoll and assayed on day four for TNP-specific IgM-PFC.

FIG. 3. Responses of high responder (D2.GD) or low responder (B10.HTT) spleen cells over a range of culture periods. Quadruplicate wells of 4×10^6 normal spleen cells from each strain were challenged with 0.1 μg/ml TNP-Ficoll and assayed on indicated days for TNP-specific IgM-PFC.

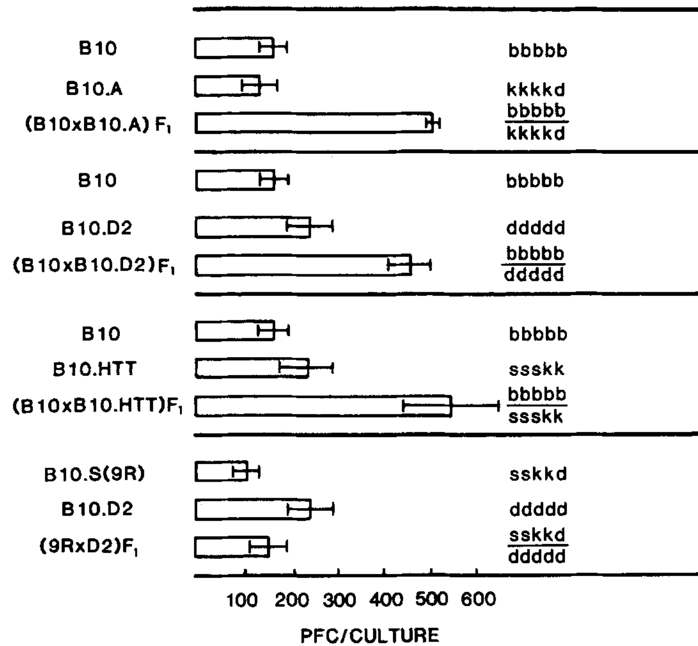

FIG. 4. Primary in vitro antibody responses of parental and F_1 hybrid animals to TNP-Ficoll. Quadruplicate wells of 4×10^6 normal spleen cells from individual mice were challenged with 0.1 μg/ml TNP-Ficoll and assayed on day four for TNP-specific IgM-PFC. Data for each experiment was normalized to a high responder (D2.GD) response of 1,000 PFC/culture of four pooled wells. Each bar represents the mean ± SEM of responses of at least six individual mice.

the F_1 tests, along with parental responses, are shown in Fig. 4. The (B10 × B10.A)F_1 is similar to the recombinant B10.A(4R) (*kbbbb*) in that it obtains the high responder K^k $I\text{-}A^k$ alleles from the B10.A parent and a complementing *b* allele to the right of the *I-E* subregion. This hybrid gives a response (512 ± 14 PFC/culture) that is equivalent to the recombinant B10.A(4R) response (503 ± 90 PFC/culture) and higher than the parental responses combined (170 ± 33 and 141 ± 36 PFC/culture). Studies of the B10.GD-like hybrid (B10 × B10.D2)F_1 suggest a similar complementation between the high responder *d* allele at the left hand locus and the *b* allele at the right hand locus of control. Response of the recombinant B10.GD (845 ± 10 PFC/culture) was higher than that of the F_1 hybrid (458 ± 43 PFC/culture).

The (B10 × B10.HTT)F_1 hybrid represents one combination of alleles that was not available to us in a recombinant haplotype. Analysis of earlier congenic and recombinant strain responses indicated that the *s* allele on the left and the *b* allele to the right resulted in high responder status in various combinations tested, and therefore should be able to complement when combined in an F_1. The high response of (B10 × B10.HTT)F_1 mice confirmed the two-gene complementation model as predicted by experiments using intra-*I* region recombinants. Finally, the low response of the [B10.S(9R) × B10.D2]F_1 confirmed the low responder status of the K^s, $I\text{-}A^s$, $I\text{-}E^d\text{-}D^d$ combination, and demonstrated that the intermediate to high response of the F_1 hybrids used in these experiments was not due to an additive effect of the parental strains, but the result of a complementation unique to the F_1 itself.

To investigate the contribution of genes outside the *H-2* region to the response to

TNP-Ficoll, responses of the B10.GD and D2.GD strains were compared. These strains are genetically identical at the *H-2* region but differ in their background genes (C57Bl/10 vs. DBA). The differences seen were not significantly different ($P > 0.2$) using the paired *t* statistic, suggesting that the background contributes little effect. Experiments are in progress to further explore this question.

Evaluation of TNP-dextran Response. To ascertain whether two-gene complementation was unique to the TNP-Ficoll response, or was a characteristic of TI-2 antigens, TNP was coupled to dextran B512 (polyglucose). The TNP-dextran response has been shown to require Ia^+ macrophages (12), is absent in mice lacking $Lyb\text{-}5^+$ B cells, and is much less dependent upon T cells than conventional thymic-dependent antigens (29). Therefore, TNP-dextran is typical of TI-2 antigens. Results of these experiments are seen in Fig. 5. The responses of various haplotypes to TNP-Ficoll and TNP-dextran were comparable. As was seen in the response to TNP-Ficoll, the D2.GD and B10.GD recombinant strains were consistently high responders, with the mean response of 24 individual D2.GD mice of 2,693 ± 292 PFC/culture. Preliminary studies of the B10.S strain in response to TNP-dextran showed a lower average number of PFC/culture (333 ± 14, $n = 2$) than in response to TNP-Ficoll (810 ± 95 PFC/culture). Although the magnitude of the B10.S response to TNP-dextran is lower, its ranking among all strains tested is consistent with its assignment as a high

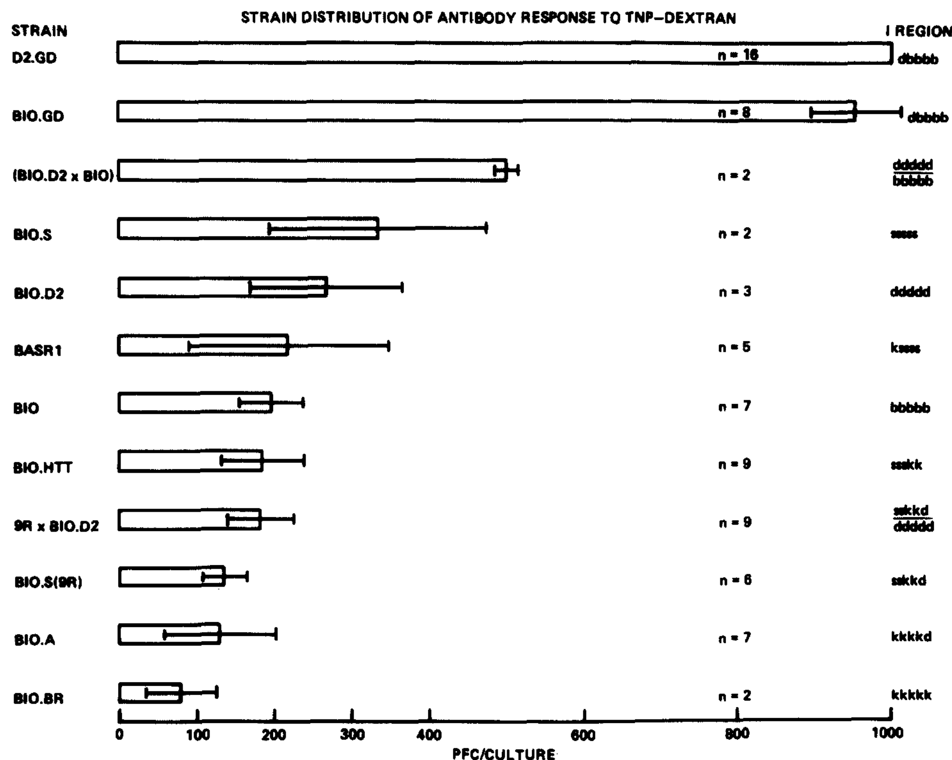

FIG. 5. Strain distribution of the primary in vitro antibody response to TNP-dextran. Quadruplicate wells of 4 × 10^6 normal spleen cells from individual mice were challenged with 10^{-6} μg/ml TNP-dextran and assayed on day four for TNP-specific IgM-PFC. Data for each experiment was normalized to a high responder (D2.GD) response of 1,000 PFC/culture of four pooled wells. Each bar represents the mean ± SEM of responses of indicated numbers of individual mice.

responder haplotype in response to TNP-Ficoll. Comparison of the remaining low responder strains demonstrates a pattern nearly identical to that of TNP-Ficoll.

In addition, responses of the recombinant strains used to map the TNP-Ficoll *Ir* gene control loci indicated that the control of the response to TNP-dextran mapped to the same areas. B10.GD mice produced an average of 955 ± 59 PFC/culture, whereas its parental haplotypes B10 and B10.D2 produced 196 ± 40 and 267 ± 100 PFC/culture, respectively, indicating *K* or *I-A* contribution to the response to TNP-dextran. Comparison of the B10.S (333 ± 140 PFC/culture) and the B10.S(9R) (135 ± 30 PFC/culture) suggested a region of control located to the right of the *I-E* subregion, as was seen in the response to TNP-Ficoll. The high response of the (B10 × B10.D2)F_1 hybrid (501 ± 16 PFC/culture) demonstrated that the two areas of control were able to complement to produce a high responder phenotype. Taken together, these observations strongly suggested that the *Ir* gene control of responses to TNP-dextran and TNP-Ficoll mapped to the same or closely linked loci. Not only were the same subregions involved in control of the anti-dextran response, but an identical pattern of high and low responder alleles was required. Preliminary experiments with a third TNP-conjugated polysaccharide, TNP-mannan, appear to be similar (data not presented).

Blocking the Response with Anti-I-A Antisera. Previous studies have shown that immune responses under specific *Ir* gene control are haplotype restricted in that (responder × nonresponder)F_1 hybrids can be inhibited by antiserum directed toward responder determinants, but not by serum directed toward nonresponder molecules (30). These results were interpreted to indicate a relationship between cell surface Ia antigens and *Ir* gene control. To determine if the *Ir* gene restriction observed in the TNP-Ficoll system was comparable to that of established antigen systems, and to determine if this restriction is expressed at the level of the antigen-presenting cell, splenic macrophages were purified, treated with anti-Ia antisera without complement, and added to purified populations of syngeneic T and B cells. These cultures were incubated with 0.1 μg/ml TNP-Ficoll, and assayed on day four for TNP-specific PFC. The results of one such experiment are shown in Fig. 6. B10.S macrophages were purified, treated with appropriate (B10.A × A.TL) anti-HTT (anti-I-A^s, B^s, J^s), or inappropriate A.TH anti-A.TL (anti-Ia^k) antisera, washed, and added to macrophage-depleted B10.S spleen cells. The PFC response was almost completely abrogated at all doses of macrophages treated with the appropriate antiserum, but gave a typical TNP-Ficoll dose curve when macrophages were treated with the inappropriate antiserum.

This experiment was repeated using the high responder recombinant D2.GD and monoclonal antibodies (Fig. 7). Splenic macrophages were blocked with the appropriate monoclonal MKD6 (anti-I-A^d) or inappropriate monoclonal antibody 10-2.16 (anti-Ia.17), washed, and combined with syngeneic T and B cells.

These results demonstrate that the pretreatment of macrophages with antibodies specific for the *I-A* subregion (anti-*I-A*^{d} monoclonal) or antiserum directed only at specificities to the right of *H-2K* (B10 × A.TL anti-HTT) was sufficient to block antigen presentation, and suggests that the left-hand locus of control maps specifically to the *I-A* subregion.

Experiments were also designed to determine the effect of antibodies directed against Ia antigens encoded in the right-hand area of *Ir* gene control. B10.A(3R) (*H-*

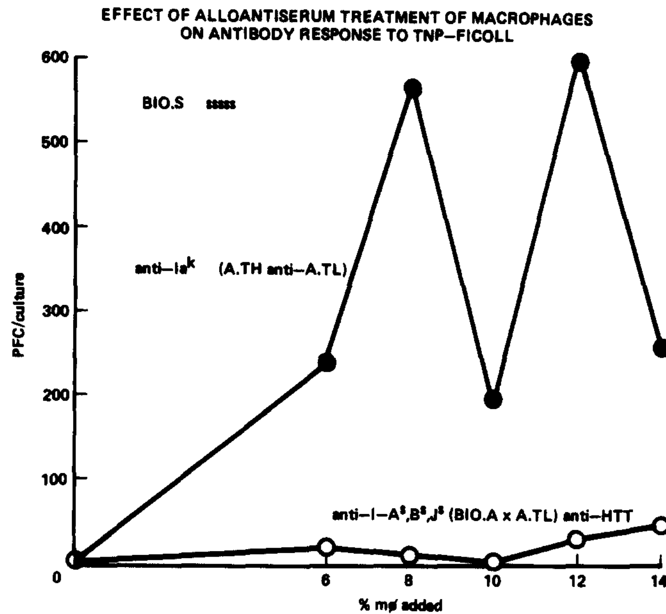

FIG. 6. Primary in vitro antibody response to TNP-Ficoll when B10.S splenic macrophages (mϕ) were briefly treated with anti-Ia alloantisera without complement. Adherent spleen cells were depleted of T and B cells, mitomycin-C treated, treated with alloantiserum, washed and added to 4×10^6 syngeneic macrophage-depleted cells and antigen. TNP-specific IgM-PFC were determined on a pooled sample of four cultures on day four.

2^{i3}) mice were challenged with cells from the C57B1/6 (*H-2^b*) strain to produce an anti-E^b, C^b, S^b, D^b alloantiserum. Pretreatment of splenic macrophages with this antiserum inhibited the TNP-Ficoll response by 68–77% in three experiments, whereas alloantiserum directed against inappropriate haplotypes in or to the right of *I-E* had no effect. Representative results are shown in Fig. 7. The ability of this serum to block antigen presentation supports the existence of an *Ir* gene located in or to the right of the *I-E* subregion that controls the response to TNP-Ficoll.

Fig. 8 illustrates that the binding of antibody to the macrophage surface does not inhibit the response to TNP-Ficoll. Macrophages from the complementing hybrid (B10 × B10.D2)F_1 were purified, pretreated with antiserum directed against either the responder gene products (I-A^d, I-E^b-D^b) or nonresponder products (I-A^b, I-E^k, C^d, S^d, D^d), and added to syngeneic T-B cells. The macrophages pretreated with antibodies specific for nonresponder determinants could support the response of the F_1 T and B cells, but blocking with antibodies that recognized determinants involved in the response significantly reduced the macrophage's ability to present antigen.

Discussion

The experiments presented demonstrate that the primary in vitro antibody response to TNP-Ficoll is under H-2-restricted *Ir* gene control. Using congenic intra-*H-2* recombinant strains, it was determined that the TNP-Ficoll response required two complementing genes. One gene controlling the response mapped in or to the left of the *I-A* subregion with high responder alleles being *s, d,* and *k*. A second subregion of control was localized in or to the right of the *I-E* subregion by the low response of

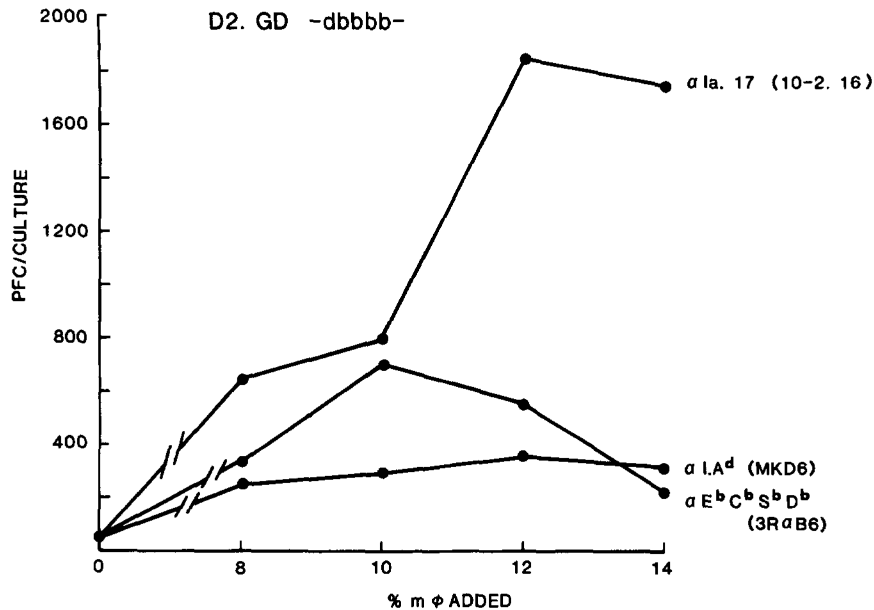

FIG. 7. Effect of monoclonal anti-*I-A* antibody and alloanti-E^b, C^b, S^b, D^b blocking treatment of D2.GD macrophages (mϕ) on the subsequent interaction of these macrophages with T-B cells. Inappropriate anti-Ia.17 (10-2.16), appropriate anti-I-A^d (MKD6), or alloantiserum B10.A(3R) anti-C57B1/6 antibodies were used without complement to pretreat splenic macrophages before culture with 4×10^6 syngeneic T and B cells and 0.1 μg/ml TNP-Ficoll. TNP-specific IgM-PFC were determined on a pooled sample of three wells on day four.

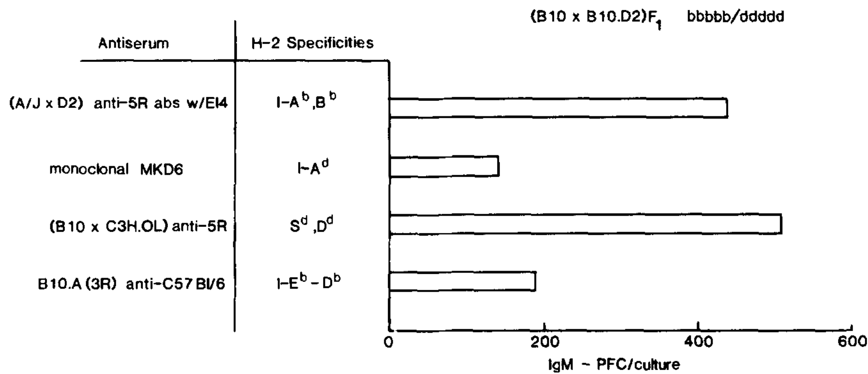

FIG. 8. Primary in vitro antibody response to TNP-Ficoll when macrophages are briefly pretreated with anti-Ia antibodies directed against responder (I-A^d, I-E^b-D^b) gene products or nonresponder products (I-A^b, I-E^k, C^d, S^d, D^d) present on responder (B10 × B10.D2)F_1 cells. TNP-specific IgM-PFC were determined on a pooled sample of three wells on day four.

B10.HTT, and required *b* or *s* alleles to produce a high response. High responder alleles at both subregions were necessary for a high responder phenotype.

The cooperation between responder alleles appeared to be a true complementation similar to that required for the GLϕ response (31). In the TNP-Ficoll response, complementing *Ir* gene control was confirmed by crossing low responder strains with a single high responder allele at one locus with strains possessing a high responder allele at the other complementing subregion. These results illustrated that high

responder recombinant haplotypes could be duplicated in F_1 hybrid animals, and indicated that there were two dominant *Ir* genes that functioned either in the *cis* or *trans* position. While low responder strains were not nonresponders, the results supported the conclusion that the high response in the F_1 hybrids was the result of a complementation unique to the F_1 itself, and not an additive effect of two independent genes.

Initially, the TNP-Ficoll response appeared similar to that of GLϕ, the random linear synthetic terpolymer of poly-(L-glu, L-lys, L-phe), where a gene mapping to *I-A* encoded the β chain of the Ia molecule, and a second gene located in *I-E* coded for the complementing E_α chain (32). The high response to GLϕ was determined by the ability to express a functional complemented product on the cell surface. In the TNP-Ficoll system, however, the high responder alleles in the right-hand region of control (*s* and *b*) have not been shown to express functional E_α chains (32). In addition, serologic analysis of alloantisera have not detected Ia determinants of the *b* and *s* haplotypes that map to the *I-E* or *I-C* subregion (20). Thus, it was difficult to implicate the I-E/C subregions in the control of the TNP-Ficoll response. It should also be noted that high responses could be obtained in appropriate F_1 hybrids despite the presence of a functional I-E product (33).

Relevant to a discussion of these results is the observation that the B10.GD (H-2^{g2}) strain, used in our experiments, contains an intragenic recombination in the sequence coding for the A_e chain (34). We mention this point in relation to the observation that the B10.GD mice responded much better than predicted by providing the responder *d* and *b* alleles in *trans* configuration in the (B10 × B10.D_2)F_1 hybrid. This difference in response when responder alleles were presented in *cis* and *trans* positions was unique to the *d-b* pair and not observed with the *k-b* or *s-b* alleles. Whether the presence of this intragenic recombination in B10.GD is related to the higher response to TNP-Ficoll will require further exploration.

As an extension of the *Ir* gene mapping studies, experiments were conducted in which purified splenic macrophages were briefly pretreated with antiserum directed against the specific *I* subregions involved in the response to TNP-Ficoll. Inhibition of antigen presentation with specific anti-Ia antibodies confirmed our assignment of *Ir* genes for this response and argues against any contribution of *H-2K*-linked genes in its control. In addition, the inhibition demonstrated that the products of these *Ir* genes are expressed on the cell surface and participate in the cell interactions involved in the response to TNP-Ficoll. The experiment presented in Fig. 6 illustrated that blocking of B10.S macrophages with (B10 × A.TL) anti-HTT serum, which recognizes the left side of the I^s region, resulted in complete loss of the TNP-Ficoll response. Similarly, when D2.GD macrophages were blocked with MKD6 monoclonal antibodies specific for I-A^d determinants (Fig. 7) the response was against inhibited.

These results confirm the involvement of the *I-A* subregion in the generation of a functional *Ir* gene product for the response to TNP-Ficoll. Localization of the right hand region of control is proving more difficult due to the lack of known Ia antigenic determinants that map to this area in the *b* or *s* haplotypes and the subsequent lack of specific alloantiserum or monoclonal antibodies directed against these regions. Experiments in which macrophages were pretreated with a broadly specific alloantiserum, B10.A(3R) anti-C57B1/6 (anti-E^b, C^b, S^b, D^b), however, showed this antiserum to be very effective in blocking antigen presentation (Fig. 7). These results indicated

that a gene product coded for by a gene in or to the right of *I-E* was required to participate in the response to TNP-Ficoll. Furthermore, the inhibition of antigen presentation by antiserum directed against the products of either subregion involved in the response strongly suggest that products from both subregions must be expressed on the cell surface to produce an immune response to TNP-Ficoll. Efforts to produce more specific probes for localization of the second *Ir* gene and investigation of the nature of its product are currently in progress.

Recent observations in our laboratory suggest an interesting possibility for the location of the right hand area of control in the TNP-Ficoll response. These preliminary experiments indicate that the second gene controlling the TNP-Ficoll response actually maps to the right of *S* and close to *H-2D*. These observations are interesting in light of those reported by Berzofsky et al. (35). These investigators have demonstrated that the genetic control of the antibody response to equine myoglobin also mapped to two complementing *Ir* genes, one in *I-A* and a second in *H-2D*. Their conclusion was reached by recombinant analysis and confirmed by testing a strain bearing a mutation in the *H-2D* region ($H\text{-}2^{dm1}$) (36). In our initial experiments, comparison of the B10.S and the recombinant B10.S(7R) responses (K^s, I^s, S^s, D^s vs. K^s, I^s, S^s, D^d) demonstrated that substitution of a low responder haplotype at *H-2D* for a high responder allele resulted in a reduction of the response. The recombination in the B10.S(7R) strain maps between the *H-2S* and *H-2D* regions (37). It is possible that this crossover event occurred at a point far enough to the left of the *D* region to include DNA of the low responder *d* haplotype, which controls the response to TNP-Ficoll but does not code for the $H\text{-}2D^d$ molecule. Experiments are in progress to further define the possible presence of the *Ir* gene located between the *S* and *D* regions and to determine whether other polysaccharides are under the same response control.

It is of interest to note that analysis of the response to a structurally different polysaccharide, TNP-dextran, revealed a pattern of control comparable to that of TNP-Ficoll. In addition, preliminary studies indicate that the response to a third TNP-polysaccharide, TNP-mannan, is also very similar. These are intriguing observations in view of the specificity seen in the control of responses to closely related protein antigens that differ at only a few residues. For example, the study of the immune response to insulins revealed that bovine, porcine, and sheep insulin display different patterns of response, although the only primary structural differences between these molecules reside in three amino acids of one chain (38, 39). Studies of the myoglobins have shown similar results (35). It is possible that the polysaccharides share a common antigenic determinant presented by the macrophage to the T helper cell. The presence of a dominant antigenic determinant is supported by previous studies which demonstrated that antibodies to a number of polysaccharides were highly cross-reactive. This cross-reactivity was due to the presence of identical portions of the structure present in each polysaccharide (40). Alternatively, it should be remembered that the examination of the antigenic determinants of the O antigens of Salmonella demonstrated that it was the sugar-side chains attached in various ways to a core polysaccharide that conferred antigenic specificity and permitted the classification of Salmonella into 40 distinct serotypes (41). With these previous observations in mind, experiments are in progress to further explore the relationship between *Ir* gene control and antigenic specificity in the TNP-polysaccharide system.

Summary

The primary in vitro antibody response to TNP-Ficoll was found to be under *H-2*-restricted *Ir* gene control. Strains B10($H\text{-}2^b$), B10.A($H\text{-}2^a$), and B10.S(9R) ($H\text{-}2^{t4}$) were consistently low responders while strains D2.GD($H\text{-}2^{g2}$), B10.GD($H\text{-}2^{g2}$), and B10.S($H\text{-}2^s$) were high responders. The in vitro TNP-Ficoll response in congenic recombinant and F_1 hybrid mice demonstrated the requirement for complementation of two independent *Ir* genes. One *Ir* gene mapped in or to the left of the *I-A* subregion with high responder alleles being *s* or *d*. The second *Ir* gene mapped to the right of the *I-E* subregion and required *b* or *s* alleles for complementation. These results were further supported by the ability to block the TNP-Ficoll response by appropriate anti-Ia serum pretreatment of the antigen-presenting macrophages. When a structurally different polysaccharide antigen TNP-dextran was used, an identical pattern of restriction was observed.

Received for publication 9 August 1982 and in revised form 3 February 1983.

References

1. Martin, J. W., P. H. Maurer, and B. Benacerraf. 1971. Genetic control of immune responsiveness to a glutamic acid, alanine, tyrosine copolymer in mice. *J. Immunol.* **107:**715.
2. Tada, T., M. Taniguchi, and C. S. David. 1977. Suppressive and enhancing T cell factors as *I* region gene products: properties and the subregion assignment. *Cold Spring Harbor Symp. Quant. Biol.* **41:**119.
3. Miller, J. F. A. P., M. A. Vadas, A. Whitelaw, and J. Gamble. 1975. H-2 gene complex restricts transfer of delayed-type hypersensitivity in mice. *Proc. Natl. Acad. Sci. USA.* **72:**5095.
4. Schwartz, R. H., M. E. Dorf, B. Benacerraf, and W. E. Paul. 1976. The requirement for two complementing *Ir-GLϕ* immune response genes in the T lymphocyte proliferative response to poly-($Glu^{53}Lys^{36}Phe^{11}$). *J. Exp. Med.* **143:**897.
5. Katz, D. H., M. Graves, M. E. Dorf, H. Dimuzio, and B. Benacerraf. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J. Exp. Med.* **141:**263.
6. Shevach, E. M., and A. S. Rosenthal. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J. Exp. Med.* **138:**1213.
7. Marrack, P., and J. W. Kappler. 1978. The role of *H-2*-linked genes in helper T-cell function. III. Expression of immune response genes for trinitrophenyl conjugates of poly-L-(Tyr, Glu)-poly-D,L-Ala-poly-L-Lys in B cells and macrophages. *J. Exp. Med.* **147:**1596.
8. Yano, A., R. H. Schwartz, and W. E. Paul. 1977. Antigen presentation in the murine T lymphocyte proliferative response. I. Requirement for genetic identity at the major histocompatibility complex. *J. Exp. Med.* **146:**828.
9. Yamashita, J., and E. M. Shevach. 1978. The histocompatibility restrictions on macrophage T-helper B-cell interaction. *J. Exp. Med.* **148:**1171.
10. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition of guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* **138:**1194.
11. Singer, A., K. S. Hathcock, and R. J. Hodes. 1979. Cellular and genetic control of antibody responses. V. Helper T-cell recognition of *H-2* determinants on accessory cells but not B-cells. *J. Exp. Med.* **149:**1208.
12. Mond, J. J., P. K. A. Mongini, D. Sieckman, and W. E. Paul. 1980. Role of T lymphocytes

in the response to TNP-AECM-Ficoll. *J. Immunol.* **125:**1066.
13. Letvin, N. L., B. Benacerraf, and R. N. Germain. 1981. B-lymphocyte responses to trinitrophenyl-conjugated Ficoll: requirement for T lymphocytes and Ia-bearing adherent cells. *Proc. Natl. Acad. Sci. USA.* **78:**5113.
14. Morrissey, P. J., H. S. Boswell, I. Scher, and A. Singer. 1981. Role of accessory cells in B cell activation. IV. Ia$^+$ accessory cells are required for the *in vitro* generation of thymic independent type 2 antibody responses to polysaccharide antigens. *J. Immunol.* **127:**1345.
15. Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell. 1977. B-lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation antigens. *J. Exp. Med.* **145:**101.
16. Mosier, D. E., B. M. Johnson, W. E. Paul, and P. R. McMaster. 1979. Cellular requirements for the primary *in vitro* antibody response to DNP-Ficoll. *J. Exp. Med.* **139:**1354.
17. Inman, J. K. 1975. Thymus independent antigens: the preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.* **114:**704.
18. Lewis, G. K., and J. W. Goodman. 1977. Carrier detected anti-hapten responses by B-cell subsets. *J. Exp. Med.* **146:**1.
19. David, C. S., D. C. Shreffler, and J. A. Frelinger. 1973. New lymphocyte antigen (Lna) controlled by the *Ir* region of the mouse *H-2* complex. *Proc. Nat. Acad. Sci. USA.* **70:**2509.
20. David, C. S. 1976. Serologic and genetic aspects of murine Ia antigens. *Transplant Rev.* **30:**299.
21. Frelinger, J. A., J. E. Niederhuber, C. S. David, and D. C. Shreffler. 1974. Evidence for the expression of Ia (H-2 associated) antigens on thymus-derived lymphocytes. *J. Exp. Med.* **140:**1273.
22. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126:**423.
23. Niederhuber, J. E., P. Allen, and L. Mayo. 1979. The expression of Ia determinants on macrophages required for the *in vitro* antibody response. *J. Immunol.* **122:**1342.
24. Niederhuber, J. E., and P. Allen. 1980. Role of *I*-region gene products in macrophage induction of an antibody response. II. Restriction at the level of T cell in recognition of *I-J* subregion macrophage determinants. *J. Exp. Med.* **151:**1103.
25. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Wash. DC).* **140:**405.
26. Bullock, W. W., and E. Moller. 1972. 'Spontaneous' B-cell activation due to loss of normal serum suppressor. *Eur. J. Immunol.* **2:**514.
27. Rittenberg, M. B., and K. L. Pratt. 1969. Antitrinitrophenyl (TNP) plaque assay: primary response of BALB/c mice to soluble and particulate immunogens. *Proc. Soc. Exp. Biol. Med.* **132:**575.
28. Boswell, H. S., S. O. Sharrow, and A. Singer. 1980. Role of accessory cells in B-cell activation. I. Macrophage presentation of TNP-Ficoll: evidence for macrophage-B cell interaction. *J. Immunol.* **124:**989.
29. Mosier, D. E., E. A. Goldings, and K. Bottomly. 1979. Activation requirements of neonatal B-lymphocytes *in vitro* and *in vivo. In* B Lymphocytes in the Immune Response. M. Cooper, D. E. Mosier, I. Scher, and E. S.Vitteta, editors. Elsevier North-Holland, Inc. New York. 91–95.
30. Schwartz, R. H., C. S. David, D. H. Sachs, and W. E. Paul. 1976. T-lymphocyte enriched murine peritoneal exudate cells. III. Inhibition of antigen induced T-lymphocyte proliferation with anti-Ia antisera. *J. Immunol.* **117:**531.
31. Dorf, M. E., and B. Benacerraf. 1975. Complementation of *H-2*-linked *Ir*-genes in the mouse. *Proc. Nat. Acad. Sci. USA.* **72:**3671.
32. Jones, P. P., D. B. Murphy, and H. O. McDevitt. 1978. Two-gene control of the expression of a murine Ia antigen. *J. Exp. Med.* **148:**925.

33. Baxevanis, C. N., Z. A. Nagy, and J. Klein. 1981. A novel type of T-T cell interaction removes the requirement for *I-B* region in the *H-2* complex. *Proc. Nat. Acad. Sci. USA.* **78:**3809.
34. Jones, P. P. 1980. Aberrant A_e (E_β) Ia polypeptide chain in $H\text{-}2^{g2}$ haplotype mice: possible result of an intragenic recombination or mutation. *J. Exp. Med.* **152:**1453.
35. Berzofsky, J. A., G. K. Buckenmeyer, and G. Hicks. 1982. Genetic control of the immune response to myoglobins. VI. Distinct *Ir*-genes for different myoglobins: complementing genes in *I-A* and *H-2D* for equine myoglobin. *J. Immunol.* **128:**737.
36. Egorov, I. K. 1974. Genetic control of *H-2* alloantigens as inferred from analysis of mutations. *Immunogenetics.* **6:**489.
37. Giotze, D. 1976. Serological characterization of Ia antigen of the $H\text{-}2^k$, $H\text{-}2^s$ and $H\text{-}2^q$ haplotypes by antisera produced against skin, lymphocytes, and lymphoblasts: strain distribution pattern of Ia antigens and their relationship to *Ir*-genes. *Immunogenetics.* **3:**139.
38. Barcinski, M. A., and A. S. Rosenthal. 1977. Immune response gene control of determinant selection. I. Intramolecular mapping of the immunogenetic sites on insulin recognized by guinea pig T and B cells. *J. Exp. Med.* **145:**726.
39. Keck, K. 1975. *Ir*-gene control of immunogenicity of insulin and A-chain loop as a carrier determinant. *Nature (Lond.).* **254:**78.
40. Heidelberger, M. 1960. Structure and immunological specificity of polysaccharides. *Prog. Chem. Org. Nat. Prod.* **18:**503.
41. Luderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of salmonella and related enterobacteriaciae. *Bacteriol. Rev.* **30:**192.