MONOCLONAL ANTIBODY CHARACTERIZATION OF A UNIQUE IMMUNE RESPONSE CONTROL LOCUS BETWEEN *H-2S* AND *D*

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Control of the immune response by genes in the *H-2* complex (*Ir* genes) has been investigated in thymic-dependent antigen systems involving synthetic polypeptides and complex protein antigens (1–4). The study of such antigens led to the conclusion that only those immune responses which require significant T cell contribution would exhibit *Ir* gene control (5). As a result, little is known about the *Ir* gene regulation of relatively T-independent antigens.

The ability to elicit an immune response to relatively T-independent type 2 antigens in nude mice suggested that the response to these antigens did not require T cell cooperation (6). In experiments using T cell lines and T cell hybrids, however, the immune response to optimal concentrations of type 2 antigens could be eliminated by T cell depletion and reconstituted with purified T cells or T cell–derived supernatant factors (7, 8). In addition, studies of the response to type 2 antigens also implicated a strict dependence on macrophages bearing major histocompatibility complex (MHC)[1] class II molecules (Ia antigens) (9–11). These observations indicated that the immune response to type 2 antigens shared many essential characteristics with conventional *Ir* gene–controlled systems and suggested that their immune response was under genetic control. Control of type 2 responses, however, also appear to involve mechanisms different than those controlling the response to classic T-dependent antigens.

We have studied the genetic regulation of a typical type 2 antigen, trinitrophenyl (TNP)-Ficoll, and have reported that the primary in vitro antibody response was regulated by two distinct loci (12). One locus of control mapped to the *I-A* subregion as determined by both recombinant analysis and blocking studies with *I-A* subregion–specific monoclonal antibodies. The second locus mapped in or to the right of the *I-E* subregion, as determined by recombinant analysis and blocking with a broadly specific alloantiserum produced in mice differing at the *I-E*, *S*, and *D* regions.

In this report, we demonstrate by recombinant analysis that the right-hand

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PFC, plaque-forming cell; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNP, trinitrophenyl; 2-ME, 2-mercaptoethanol.

locus of immune response control is located between the *H-2S* and *D* regions. In addition, a monoclonal antibody directed against this right-hand region of control has been produced (48-21.7) that blocks the response to TNP-Ficoll at the level of the antigen-presenting cell. The monoclonal antibody 48-21.7 is specific for the high responder *b* allele at the right-hand locus and does not inhibit responses to other protein antigens tested. The immune response to TNP-Ficoll is not inhibited by monoclonal antibodies that react with $H\text{-}2D^b$ or Qa-2 specificities, suggesting that the TNP-Ficoll response is controlled by a unique locus located between *H-2S* and *D*. Finally, 48-21.7 recognizes and precipitates an unique product of ~40,000 mol wt that is distinct from the $H\text{-}2D^b$ region product recognized by the monoclonal antibody B22/249.

Materials and Methods

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

Antigens. TNP-aminoethyl carbamethyl (AECM)-Ficoll (conjugation ratio, TNP_{34}-AECM-Ficoll), was obtained from Biosearch Laboratories, San Rafael, CA. TNP-bovine gamma globulin (TNP-BGG) was purchased from Calbiochem-Behring Corp., San Diego, CA, and contained 61 TNP groups per molecule of BGG.

Antisera. Restricted anti-Ia alloantisera were prepared as previously described (13–15). Batches of serum from several bleedings were tested in a dye exclusion microcytotoxicity assay for appropriate anti-Ia reactivity and antibody titer (15). The hybridoma cell lines MKD6 (anti-I-A^d) and MKS4 (anti-I-A^s) were gifts from Dr. Phillippa Marrack, National Jewish Hospital, Denver, CO. The cell line producing monoclonal antibodies directed against specificity Ia.17 (10-2.16) was obtained from the Salk Cell Distribution Center, La Jolla, CA. Monoclonal antibody Y-7KB (anti-Qa-2^a) was a gift from Dr. Donal Murphy, Yale University, New Haven, CT.

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 (16) with 10% fetal calf serum (FCS) (Reheis Co. Inc., Phoenix, AZ). Cultures were established in 24-well plates (Costar, Data Packaging Corp., Cambridge, MA). Antigen was added at a final concentration of 0.1 μg/ml TNP-Ficoll or 5 μg/ml TNP-BGG in a final volume of 1 ml/well. Plates were incubated for 4 d at 37°C in 7% CO_2.

For experiments requiring purified cell preparations, separation of the cells was accomplished using previously published procedures (17, 18). Briefly, for peritoneal macrophage isolation, mice were injected intraperitoneally with 45 μg of concanavalin A (Con A) (Pharmacia Fine Chemicals, Piscataway, NJ) in 1.5 ml phosphate-buffered saline (PBS). 3 d later, peritoneal exudate cells were harvested by flushing the peritoneum with cold tissue culture medium containing 0.1 M Hepes and 10% FCS. Cells were pelleted, resuspended, and the number of viable cells determined. These cells have been demonstrated to be ~70% Ia^+ macrophages. Purified macrophages were seeded in 24-well Costar plates at the desired number of Ia^+ macrophages; usually 5% of the number of macrophage-depleted cells were added per well. The cells were allowed to adhere for 2 h, washed to remove nonadherent cells, treated with mitomycin C (Sigma Chemical Co., St. Louis, MO), and extensively washed. In antibody pretreatment experiments, 0.5 ml of appropriately diluted antibody was added per well; macrophages were then incubated for 45 min and washed four times with fresh medium.

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 popu-

lation. Total cell loss with this procedure was 40–50%, but no difference in the T cell/B cell ratio could be discerned.

Controls for each experiment included: macrophage-depleted spleen cells plus antigen; untreated macrophages plus antigen; and macrophages pretreated with antibody plus antigen. These controls produced no plaque-forming cells (PFC) in any experiment.

Local Hemolysis in Gel. TNP-specific, IgM antibody-producing cells were assayed on day 4 of the culture period by a modified Jerne technique (19, 20). Sheep erythrocytes were lightly conjugated with TNP and used to determine the number of direct PFC (21). Greater than 90% of PFC were inhibited by 10^{-2} M TNP-lysine. Results were corrected for background PFC using nohaptenated sheep erythrocytes.

Production of Antibody-secreting Hybrid Cell Lines. 6–8-wk-old female B10.A(3R) mice were initially injected with 2×10^7 lymphoid cells from C57BL/6 mice both intraperitoneally and subcutaneously, followed 1 wk later by an intravenous injection. After six injections, the mice were bled and the serum tested for activity. 3 d after the last injection, when the serum had the desired activity, the mice were boosted and the immune spleen cells were fused with P3X63-Ag8.653 myleoma cells (22–24).

Fine Specificity Testing. Clones that were positive for antibody production were screened for their ability to block the in vitro primary antibody response to TNP-Ficoll in the high responder D2.GD strain. Positive clones were subcloned twice by limiting dilution and tested for maximum inhibitory capacity. Supernatants from expanding cultures were collected, processed, and stored at −20°C. In addition, high titer antibody was produced by inducing ascites tumors in [BALB/c × B10.A(3R)]F_1 mice primed with pristane (2,6,10,14-tetramethylpentadecane, 96%) (Aldrich Chemical Co., Milwaukee, WI). Monoclonal antibody 48.21, an IgM class antibody, was purified by Sephacryl S-300 gel filtration of the 40% ammonium sulfate fraction of ascites. Purity of the IgM preparation was determined by SDS-PAGE.

Radiolabeling and Precipitation of Antigens. The procedures for radiolabeling mouse spleen lymphocytes and for extracting and immunoprecipitation of antigens are modifications of those described by Jones (25). Briefly, splenic lymphocytes were incubated for 4–5 h at 25×10^6 cells/ml in methionine-free RPMI 1640 medium containing 5% FCS and 250 μCi/ml [^{35}S]methionine/ml (1,000 mCi/mmol; New England Nuclear, Boston, MA). Membrane proteins were extracted from washed cells with 0.5% Nonidet P-40 (Bio-Rad Laboratories, Richmond, CA) buffer. Immune complexes were removed from lysates by centrifugation at 15,000 *g* for 15 min and incubation for 3 h with Staphylococcus protein A–coupled Sepharose beads (Staph A beads) (Pharmacia Fine Chemicals, Piscataway, NJ).

Antigens were immunoprecipitated from the extracts with either supernatants from antibody-producing hybridomas or purified ascites. Lysates were incubated with the antibody overnight, followed by the addition of Staph A beads or, for IgM antibodies, Staph A beads complexed with rabbit anti–mouse IgM, A, and G (Accurate Chemical & Scientific Co., Westbury, NY). The bound proteins were eluted from the Staph A with electrophoresis buffer containing sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories) and 2-mercaptoethanol (2-ME) (Aldrich Chemical Co.) for reducing gels or without 2-ME for nonreducing analysis.

SDS-PAGE Analysis. Precipitated proteins were separated in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels according to standard procedures. Separating gels contained 12% acrylamide (Bio-Rad Laboratories) with 0.5% *N,N*-diallyltartardiamide (DATD) crosslinker and 0.1% SDS. Stacking gels contained 4% acrylamide; 25 μl vol protein samples were added to each lane and gels were electrophoresed at 25 mA/gel. They were then fixed in 30% methanol, 10% acetic acid, 10% TCA; enhanced in 1.0 M sodium salicylate, dried, and autoradiographed for 4 d at −70°C.

Results

We have previously reported (12) that the primary immune response to TNP-Ficoll was regulated by two distinct complementing loci within the murine *H-2*

complex. The first locus was found to map within the *I-A* subregion and required the *d* and *s* alleles for the high responder and the *k* allele for the intermediate responder phenotype. A second region of control was defined in or to the right of the *I-E* subregion with *b* or *s* as the high responder alleles. The presence of high responder alleles at both loci was required for maximum response to TNP-Ficoll. Finally, high responses obtained in complementing (low responder × low responder)F_1 hybrid animals confirmed the dual locus regulation of the response, and demonstrated that these distinct loci complement in the *trans* as well as the *cis* configuration.

Analysis of responses of recombinant strains B10.S(7R) and B10.A(2R) allowed a more precise definition of the right-hand locus of control (Fig. 1). The B10.S(7R) strain (K^s, I^s, S^s, D^d) is identical to the high responder B10.S (K^s, I^s, S^s, D^s) strain with the exception of the low responder *d* allele at the *H-2D* region. If the right-hand locus controlling the TNP-Ficoll response mapped within the *I* region, the B10.S(7R) recombinant would produce a response equivalent to that of the B10.S strain. Challenge of B10.S(7R) recombinant strain, however, resulted in significantly lower numbers of TNP-specific plaques per culture and implicated an area of control outside of the *I* region, very close to or within the *H-2D* region.

The response of the B10.A(2R) (K^k, I^k, S^d, D^b) strain was assayed in an effort to determine the role of the *H-2D* locus in the TNP-Ficoll response. This recombinant is identical to the low responder B10.A strain but expressed a high responder *b* allele at *H-2D*. If the right-hand locus of control mapped in *H-2D*, the B10.A(2R) recombinant would produce a response equivalent to the (B10.A × B10)F_1 ($K^{k/b}$, $I^{k/b}$, $S^{d/b}$, $D^{d/b}$), (520 ± 90 PFC/culture) that expresses the same alleles at the relevant loci. Challenge of the B10.A(2R) strain resulted in a low response equivalent to that of the B10.A parental strain. This result indicated that the crossover in the B10.A(2R) recombinant occurred to the right of the gene controlling the TNP-Ficoll response, and, consequently, these mice fail to express the high responder *b* allele at the right-hand locus. Therefore, the

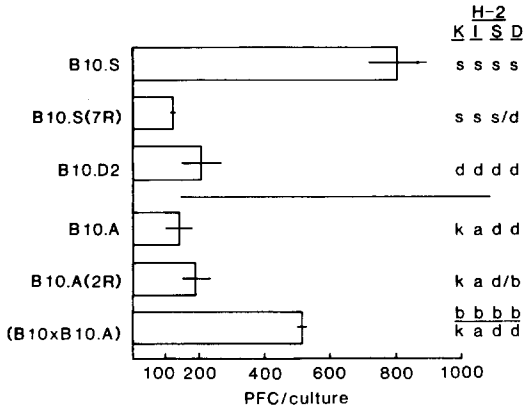

FIGURE 1. Assay of recombinant strains with crossover events occurring between the *H-2S* and *D* regions. Quadruplicate wells of 4×10^6 normal spleen cells were challenged with 0.1 μg/ml TNP-Ficoll and assayed on day 4 for TNP-specific IgM PFC. Responses of each strain were normalized to the high responder D2.GD response of 1,000 PFC/culture.

responses of the B.10S(7R) and B10.A(2R) strains suggest a locus of immune response control located between the *S* and *D* regions, bounded on the left by the crossover event in the B10.S(7R) recombinant and on the right by the crossover event in the B10.A(2R) strain.

Because the strains mapping the right-hand locus of control were of different haplotype combinations, it was possible that the differences in response observed between the B10.S(7R) and the B10.A(2R) strains were due to differences in genetic organization of the two haplotypes (*s* vs. *a*) rather than differences introduced by recombination. To eliminate this possibility, three congenic strains (developed by Dr. Shreffler, Washington University, St. Louis, MO) with recombination occurring between the *H-2S* and *H-2D* regions were studied. Each of these strains was independently derived from a (B10 × B10.A) parental cross and identified as a recombinant by serological analysis (26). The results of challenging these strains with TNP-Ficoll and the control antigen TNP-BGG are shown in Table I. The $I\text{-}A^k$, S/D^b combination of alleles produced an intermediate response of ~500 PFC/culture in response to TNP-Ficoll, a level of response observed in the B10.A(4R) and (B10 × B10.A)F_1 strains.

The combination of $I\text{-}A^k$, S/D^d resulted in a low responder phenotype as seen in the B10.A (Fig. 1) and B10.A(2R) strains (140 ± 36 and 190 ± 36 PFC/culture, respectively). Thus, if the recombination in a strain had occurred to the right of the gene controlling the response to TNP-Ficoll, the strain would express low responder alleles at both loci, and resulted in a response equivalent to B10.A(2R) mice. Alternatively, if the crossover in the particular strain occurred to the left of this gene, the strain would express the high responder *b* allele at this right-hand locus and show an intermediate response equivalent to B10.A(4R) mice.

Challenge of the new recombinant strains produced the results shown in Table I. Each of the strains produced equivalent responses to the control antigen TNP-BGG. The B10.BAR11 and B10.BAR6 strains produced low responses to TNP-Ficoll, indicating indentity with the B10.A(2R) strain at the right-hand locus. In contrast, the B10.BAR10 strain showed intermediate responses, like that of B10.A(4R) mice. Therefore, comparison of responses in the B10.BAR6,

TABLE I

Comparison of Antibody Responses to TNP-Ficoll in Strains with Crossovers Between the H-2S and H-2D Regions

Strain	K	I-A/I-E	S	D	TNP-Ficoll*	TNP-BGG
B10.BAR6	k	k/k	d	b	148 ± 32	240 ± 63
B10.BAR10	k	k/k	d	b	425 ± 191	320 ± 70
B10.BAR11	k	k/k	d	b	110 ± 36	275 ± 75
B10.A(2R)	k	k/k	d	b	190 ± 36	ND
B10.A(4R)	k	k/b	b	b	512 ± 140	380 ± 163
(B10 × B10.A)F_1	b	b/b	b	b	502 ± 91	279 ± 86
	k	k/k	d	d		

*Results are expressed as PFC/culture of four pooled wells of 4×10^6 spleen cells. In each experiment at least three mice from each strain were tested. Data of four experiments are normalized to a high responder D2/GD response of 1,000 PFC/culture. ND, not done.

B10.BAR10, and B10.BAR11 strains defined a locus controlling the response to TNP-Ficoll in a single haplotype, bounded on the left by the crossover event in the B10.BAR10 and on the right by the crossover event in the B10.BAR6 and B10.BAR11 strains.

Antibody Blocking of the Response to TNP-Ficoll. Previous studies (12) have demonstrated that the response to TNP-Ficoll is significantly inhibited by pretreatment of complementing (low responder × low responder)F_1 macrophages with antiserum directed toward high responder determinants of either locus, while antibodies directed toward the corresponding low responder determinants have no effect. These results indicated that antibodies directed toward specific determinants could be used in functional assays to identify those cell surface antigens directly involved in controlling the response to TNP-Ficoll. To confirm the recombinant analysis of the TNP-Ficoll response and to investigate the role of the *H-2D* region in its control, antibody blocking studies were performed. Con A–induced peritoneal exudate cells were used as a source of macrophages in these experiments. These cells were purified and pretreated with either alloantiserum or monoclonal antibodies and mitomycin C, washed extensively, and cultured with syngeneic carbonyl iron–treated spleen cells and antigen as described in Materials and Methods.

Initial blocking experiments showed that both alloantiserum and monoclonal antibodies directed against high responder I-A specificities effectively blocked the response to TNP-Ficoll. Blocking of the right-hand region of control was demonstrated by inhibition of the response with the broadly specific B10.A(3R) anti-C57BL/6 alloantiserum, which contained antibodies directed against membrane-expressed gene products of the *I-E* through *H-2D* segment of the high responder *b* haplotype (Fig. 2). The anti-D^b activity of this antisera could be absorbed with EL-4 cells that bear class I but not class II molecules. Absorption of anti-D^b activity was confirmed by cytotoxic tests (data not shown). As shown in Fig. 2, the ability of this antiserum to block the TNP-Ficoll response is unaffected by removal of anti-D^b activity.

To produce a specific probe to the right-hand locus of control, spleens from mice producing the B10.A(3R) anti-C57BL/6 alloantiserum (anti-E^b-D^b) were fused with the P3X63Ag8.653 myeloma (22). Antibodies produced by the resulting hybridomas were screened for their ability to block the D2.GD response to TNP-Ficoll. The results of the screening of two different subclones of monoclonal antibody 48-21 are shown in Fig. 3. Pretreatment of macrophages with antibodies from either 48-21.7 or 48-21.4 subclones resulted in inhibition equivalent to that obtained with B10.A(3R) anti-C57BL/6 alloantiserum.

Further characterization of the specificity of monoclonal antibody 48-21.7 included assessment of its effect on responses to other antigens. Fig. 4 illustrates that 48-21.7 did not alter the primary antibody responses of high responder D2.GD animals to the T-dependent antigen TNP-BGG. Pretreatment of macrophages with 48-21.7 blocked the TNP-Ficoll response by 62%, yet with identical pretreatment it had no effect on the primary response to TNP-BGG. In addition, pretreatment of macrophages with 48-21.7 had no effect on secondary responses to either LDH-B or MOPC-173 as measured in the T cell prolif-

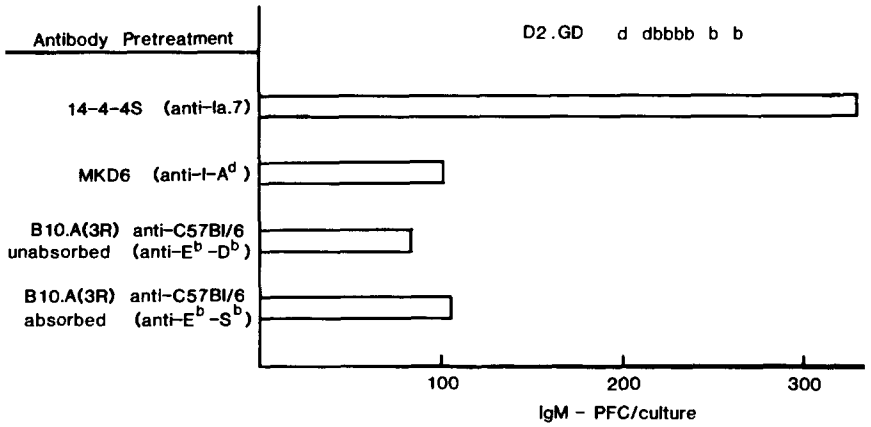

FIGURE 2. Antibody blocking of the primary response to TNP-Ficoll. D2GD macrophages were pretreated with antibodies appropriate for the I-A subregion products (MKD6), alloserum specific for *I-E* through H-*2D* regions (unabsorbed); serum with *H-2D* specificities removed (absorbed); or inappropriate monoclonal antibodies (14-4-4S).

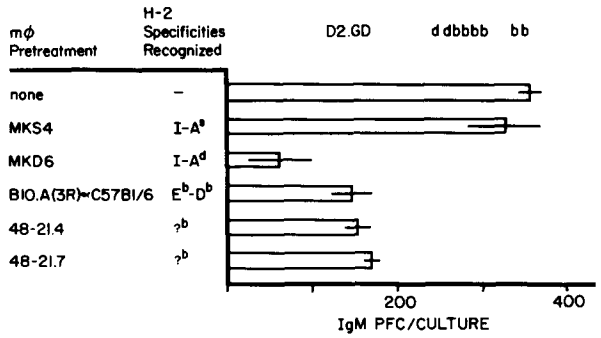

FIGURE 3. Primary in vitro antibody response to TNP-Ficoll when Con A–induced D2.GD peritoneal macrophages are briefly pretreated with two different subclones of the hybridoma 48-21. Macrophages were purified and incubated with medium, inappropriate anti-I-A^s antibodies (MKS4), appropriate anti-I-A^d (MKD6), alloantiserum B10.A(3R) anti-C57BL/6, supernatant of subclone 48-21.4, and 48-21.7. 4×10^6 macrophage-depleted cells and antigen were added. On day 4, TNP-specific plaques were determined on a pooled sample of three cultures.

eration assay (data not shown). These results strongly suggest that the monoclonal antibody 48-21.7 recognized a specificity unique to the TNP-Ficoll response.

Similarly, Fig. 5 illustrates that blocking by 48-21.7 was haplotype specific. High responder B10.S (K^s, I^s, S^s, D^s) macrophages were purified and pretreated with the inappropriate anti-I-A^d (MKD6) antibodies, or the appropriate anti-I-A^s (MKS4) antibodies. Test antisera were the B10.A(3R) anti-C57BL/6 alloantiserum, which recognizes the right-hand region of the *b* haplotype, or monoclonal antibody 48-21.7. The inability of the 48-21.7 monoclonal antibody to block the TNP-Ficoll response in the high responder *s* haplotype indicated that allelic forms of the high responder determinant may exist.

In an effort to rule out an *H-2D* region contribution to the control of the response to TNP-Ficoll, D2.GD macrophages were pretreated with a monoclonal antibody (B22/249) that specifically recognized the class I determinant *H-2D^b*

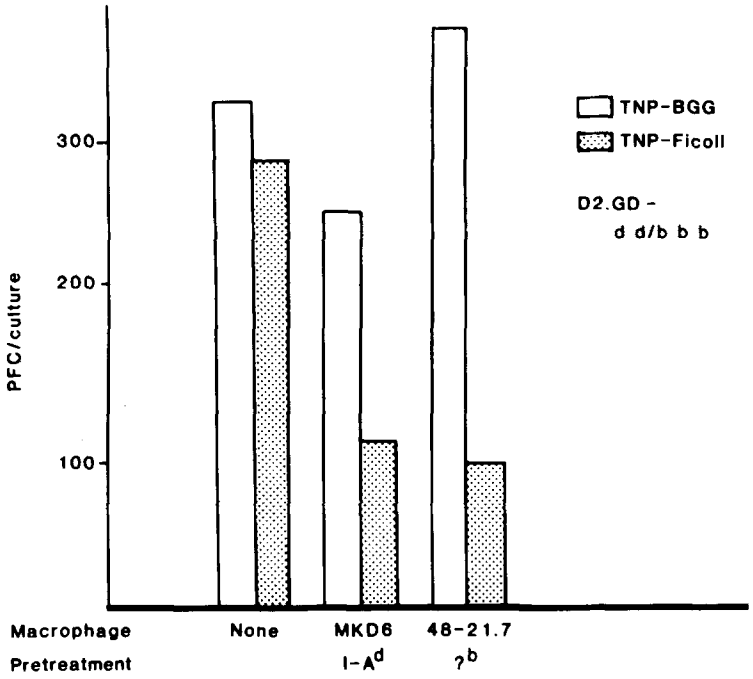

FIGURE 4. Blocking function with monoclonal antibody 48-21.7 is antigen specific. Purified peritoneal macrophages from D2.GD mice were pretreated with medium, anti-I-A^d (MKD6) antibodies, or 48-21.7. Syngeneic T-B cells were added and cultures were challenged with TNP-BGG (open bars) or TNP-Ficoll (shaded bars). Cultures were assayed for primary IgM PFC on day 4.

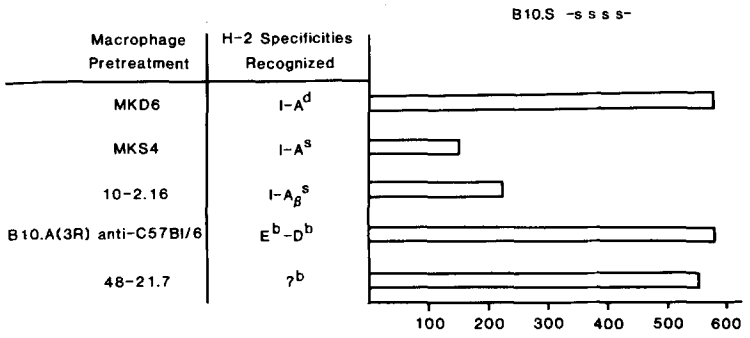

FIGURE 5. Blocking of macrophage function with monoclonal antibody 48-21.7 is haplotype specific. Purified peritoneal macrophages from B10.S mice were pretreated with inappropriate anti-I-A^d antibodies (MKD6), appropriate anti-I-A^s antibodies (MKS4), anti-Ia.17 (10-2.16), or alloantiserum B10.A(3R) anti-C57BL/6 or 48-21.7. Syngeneic T-B cells were added with antigen. Cultures were assayed for primary IgM PFC on day 4.

(27, 28). Fig. 6 illustrates that macrophage pretreatment with monoclonal antibody B22/249 had no effect on the response to TNP-Ficoll. In addition, this result indicates that binding of the antibody B22/249 to the D2.GD macrophage cell surface, as demonstrated by cytotoxic tests, is not sufficient to inhibit their ability to generate a TNP-Ficoll PFC response.

It has been suggested (29) that quantitative variation in the amount of Qa-1

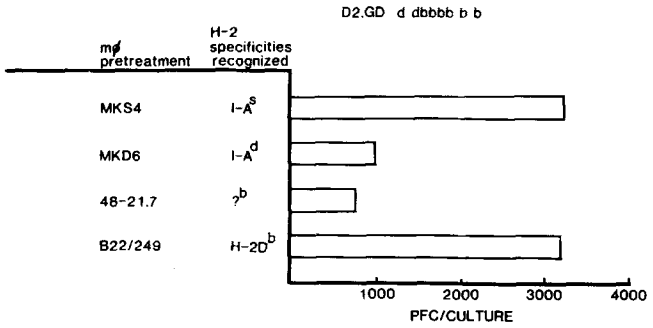

FIGURE 6. Anti-H-2D^b monoclonal antibodies do not block the primary response to TNP-Ficoll. D2.GD macrophages were purified, pretreated with inappropriate anti-I-A^s (MKS4) antibodies, appropriate anti-I-A^d (MKD6) antibodies, 48-21.7, or H-2D^b-specific monoclonal antibody B22/249, washed, and cultured with 4 × 10^6 syngeneic T-B cells and antigen. Cultures of three pooled wells were assayed on day 4.

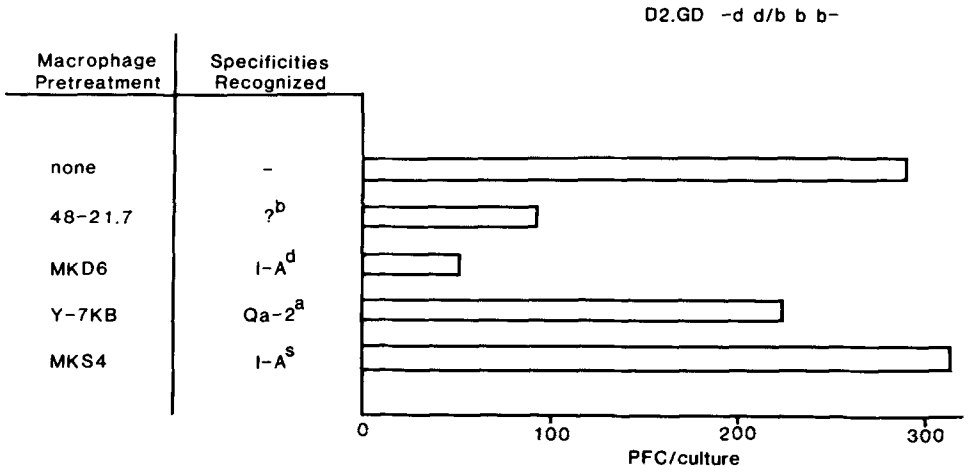

FIGURE 7. Anti-Qa-2^a–specific monoclonal antibodies do not block the primary response to TNP-Ficoll. D2.GD macrophages were purified, pretreated with inappropriate anti-I-A^s (MKS4) antibodies, appropriate anti-I-A^d (MKD6) antibodies, 48-21.7, or Qa-2^a-specific monoclonal antibody Y-7KB, washed, and cultured with 4 × 10^6 syngeneic T-B cells and antigen. Cultures of three pooled wells were assayed on day 4.

antigen expressed on the cell surface is controlled by a gene within or very close to *H-2D*. *Qa-2* expression has been postulated to be controlled by a similar gene in an analogous manner (30). This hypothesis raised the question whether control of the TNP-Ficoll response by the right-hand locus was mediated through the quantitative expression of a Qa antigen rather than the qualitative expression of a unique molecule. To test this possibility, macrophages were pretreated with monoclonal antibodies specific for a *Qa-2* region–controlled determinant, Y-7KB (Fig. 7). Qa-2 is the predominant Qa antigen found on cells other than T lymphocytes (31). If 48.21 either recognized a Qa-2 molecule or the product of the gene which regulated the quantitative expression of Qa-2, then Y-7KB should produce results similar to those obtained with 48-21.7. The failure of the anti-Qa-2 reagent to inhibit the TNP-Ficoll response demonstrated that the Qa-2

specificity recognized by this antibody was not involved in the response, and supported the conclusion that 48-21.7 recognized a unique specificity encoded between *S* and *D*.

Characterization of the Product Recognized by 48-21.7. Initial characterization of the product recognized by the monoclonal antibody 48-21.7 by immunoprecipitation of endogenously [^{35}S]methionine-labeled whole spleen lysates from D2.GD is shown in Fig. 8. 12% SDS-PAGE analysis resolved a product of 40,000 (40 K) mol wt precipitated by the monoclonal antibody 48-21.7. This band is slightly smaller than the band precipitated with the anti-H-2D^b monoclonal B22/249. To determine whether 48.21 and B22/249 could be recognizing different forms of the same molecule, sequential immunoprecipitations were performed. As can be seen from Fig. 8*b*, when D2.GD splenic lysates were cleared of any material reactive with B22/249 and then tested for the presence of material reactive with 48-21, the 40 K mol wt band was observed (lane *B*). When the reverse was done, that is, when lysates were cleared of 48-21-reacting material and the resulting cleared lysate was precipitated with B22/249, a 43 K mol wt

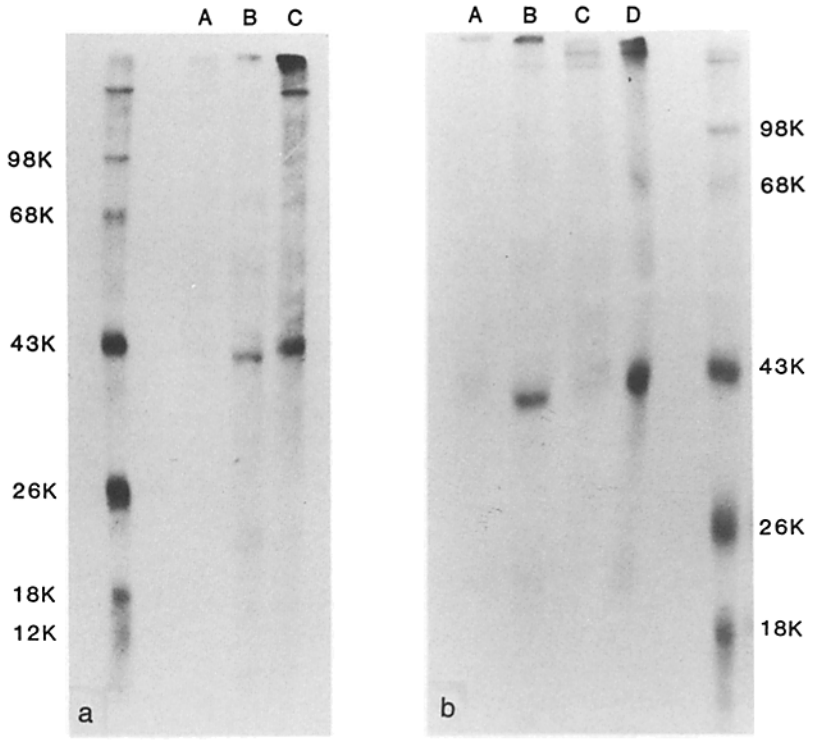

FIGURE 8. (*a*) D2.GD immunoprecipitates in reducing 12% SDS-PAGE. D2.GD splenic cell lysates (*A*) immunoprecipitated with inappropriate antibody anti-I-A^s (MKS4), (*B*) immunoprecipitated with 48.21.7, (*C*) immunoprecipitated with B22/249 (H-2D^b). (*b*) Sequential immunoprecipitates of D2.GD splenic lysates with 48-21.7 and B22/249 in reducing 12% SDS-PAGE. (*A*) First precipitation with B22.249, second precipitation with the same B22/249 antibody. (*B*) First precipitation with B22/249, second precipitation with 48.21.7. (*C*) First precipitation with 48.21.7, second precipitation with 48.21.7. (*D*) First precipitation with 48.21.7, second precipitation with B22/249.

band was observed (Fig. 8*b*, lane *D*). Control lanes *A* and *C* demonstrate the completeness of removal of B22/249-(*A*) and 48.21-(*C*) reactive material from the first precipitation. These results indicate that the molecule recognized by 48.21 is distinct from the H-2D^b molecule.

Discussion

The experiments presented demonstrate that the primary in vitro antibody response to TNP-Ficoll is controlled by two complementing loci. Recombinant analysis mapped one locus to the *I-A* subregion, and the second locus between the *S* and *D* regions. Other investigators (32) have also found evidence for immune response control loci outside of the *I* region and close to the *D* end of the *H-2* complex. Berzofsky et al. (2) showed that the antibody response to equine myoglobin was controlled by two complementing genes, one in *I-A* and a second in *H-2D*. This conclusion was reached by recombinant analysis and confirmed by testing strain *H-2^{dml}*, which has a mutation in the *H-2D* region (33). In addition, a genetic region between *S* and *D*, designated *H-2U* by O'Neill and Parish (34), was identified by alloantisera produced in strains that differed at the *D* region. This allosera retained activity against several target strains after absorption to remove *D*-specific antibodies. The *H-2U* region appears to control antigens similar in their cellular distribution and molecular weight to Ia antigens. Krco et al. (35) demonstrated that the control of the T cell proliferative response to hemoglobin mapped to two separate *Ir* genes, one of which mapped to the D end of the *H-2* complex. Recombinant strains challenged with intact hemoglobin or the alpha subunit demonstrated *I-A* region control (35), whereas challenge with the beta chain subunit exhibited control mapping to the *H-2D* region.

In addition to the evidence placing *Ir* genes between the *S* and *D* regions, other evidence has mapped genes controlling the phenotypic expression of certain enzymes to the region near the *H-2D*. For example, *Neu-1*, a locus controlling the activity of the enzyme neuraminidase, which alters the sialation of at least five different enzymes, has been closely linked to the *D* region (36, 37). Variants of these enzymes were originally thought to be controlled by separate loci mapping to *H-2*, but now variants appear to be the result of different alleles of the *Neu-1* locus (38). It is important to note, however, that the patterns of allelic variation of *Neu-1* do not follow the patterns of high and low response established for the response to TNP-Ficoll. For example, C57BL/10 and BALB/c strains both have the *Neu-1^b* allele but C57BL/10 carries the TNP-Ficoll high responder *b* haplotype, and BALB/c, the low responder *d* haplotype. It is unlikely, therefore, that the *Neu-1* locus is involved in the regulation of the Ficoll response.

The existence of multiple genes mapping to the right end of the MHC, and the presence of genes that control or modify the expression of other molecules on the cell surface, raised the possibility that the product of the right-hand locus which controls the response to TNP-Ficoll did not function in a manner similar to an Ia antigen. In fact, the molecular weight of the product recognized by monoclonal antibody 48-21.7 indicated that this could be the case. The 40 K mol wt protein, immunoprecipitated with 48-21.7, was more similar to that of a class I or class I–related (Qa, Tla) gene product than a typical class II gene product.

Molecular data has shown that the *d* haplotype contains 36 distinct class I genes (39). 31 of the 36 class I genes have been mapped to the *Qa* and *Tla* regions of the MHC, while the other five map to *H-2K* and *D* regions (40). Studies of the *b* haplotype have indicated 23 of 26 class I genes in this haplotype to map to the *Qa-2,3* and *TL* regions (41). To date, the function and expression of the products of most of these genes is unknown.

The presence of genes in the region between *H-2S* and *Tla* that control the quantitative expression of the class I–related antigens Qa-1 and, perhaps, Qa-2, has raised the possibility that Qa-1 or Qa-2 were in someway responsible for the different levels of response to TNP-Ficoll (29, 30). Quantitative as well as qualitative differences in expression of Qa-1 specifities in certain mouse strains have been demonstrated by variations in cytotoxicity and absorption of antiserum activity. For example, $H\text{-}2D^k$ strains express less of the Qa-1 antigen than other strains (29). Similarly, immunochemical and serological studies demonstrated that different strains expressed varying amounts of the Qa-2 antigen, and recombinant analysis suggested that these quantitative differences were due to modifying genes located to the right of the *S* region, distinct from the *Qa* structural genes (30, 42, 43). In some instances, quantitative variation in expression of class I molecules has been shown to influence function. For example, the amount of H-2D product on the cell surface correlates with resistance to virus infection (44) and with immunity against virus-infected cells (45). However, it is unlikely that Qa or H-2D molecules are involved in regulation of the immune response to TNP-Ficoll.

The strongest evidence against involvement of Qa-2 molecules in the regulation of the TNP-Ficoll response was the failure of the anti-Qa-2 monoclonal antibody to block the response. Of course, the failure of a single anti-Qa-2 antibody to inhibit the response to TNP-Ficoll does not rule out the possibility that other Qa molecules play a role. However, if 48.21.7 recognizes either a $Qa\text{-}2^a$ determinant or the product of the gene, located between *H-2D* and *Tla*, that regulates the quantitative expression of Qa-2, then the anti-$Qa\text{-}2^a$ antibody Y-7KB would be expected to block the immune response.

Experiments in which responding cells were pretreated with antibody and complement demonstrated that the specificity recognized by 48-21.7 was not present on a T or B cell population critical to the TNP-Ficoll response. In contrast, treatment of the T-B populations with the Y-7KB monoclonal antibody (anti-$Qa\text{-}2^a$) and complement resulted in significant elimination of those cells necessary for this response (data not shown). Similar results have been reported by Kincade et al. (31) in which a polyvalent Qa-2 alloantiserum demonstrated the presence of Qa on cells capable of primary immune responses to TNP-Ficoll. Taken together, the failure of the anti-Qa-2 antibody to block the TNP-Ficoll response and the different tissue distribution of 48-21.7 and Y-7KB appear to preclude the possibility that 48-21.7 monoclonal antibody recognizes a Qa specificity. Sequential immunoprecipitation studies with 48.21 and Y-7KB are in progess.

Blocking studies were extended to include monoclonal antibodies specific for determinants on the class I $H\text{-}2D^b$ region molecule and demonstrated that products of the $H\text{-}2D^b$ region do not participate in the antibody response to

TNP-Ficoll. The alloantiserum containing anti-D^b antibodies showed equivalent blocking ability before and after class I specificities were removed by absorption. In addition, pretreatment of macrophages with monoclonal antibody B22/249 specific for *H-2D*^{b} showed no inhibition of the TNP-Ficoll response. In more recent experiments we have used another monoclonal antibody with *H-2D*^{b} specificity (28-14-8) with similar result.

Melino et al. (46), in studies directed at determining the molecular heterogeneity of the *H-2D*^{b} region, used sequential immunoprecipitations with monoclonal antibodies 28-14-8 and B22/249, and anti-D^b alloantisera. The sequential immunoprecipitations supported the existence of only one gene product in this region. Others (47), however, have reported evidence for at least three serologically detectable molecules controlled in the *H-2D*^{b} locus. At the DNA level, only one D^b gene was identified in overlapping cosmid clones of this region (41). Thus, the failure to inhibit the response using anti-D^b antibodies, and the precipitation of two distinct molecules, support the conclusion that the 48.21 antibody does not recognize a class I H-2D^b molecule.

Our results are consistent with the hypothesis that monoclonal antibody 48-21.7 recognizes the product of a novel class I–related gene that is located between the *S* and *D* loci. Immunoprecipitation analysis strongly suggests that the product recognized by 48-21.7 was distinct from either the class I *H-2D*^{b} molecule or an Ia molecule. We do not know for certain whether the 48.21.7 molecule is associated with β_2-microglobulin; reduced SDS-polyacrylamide gels occasionally show a very faint band at 12 K mol wt. Since we have seen variable amounts of β_2-microglobulin precipitated with our D^b reagents, it is possible that an association between β_2 and the 48.21.7 molecule occurs but is of lower affinity than most class I molecules. The observation that there may be no association of the product recognized by 48-21.7 and β_2-microglobulin may have functional significance. In an earlier study (48), cells transfected with clones of class I genes were assayed for an increase in β_2-microglobulin as an indicator of foreign class I antigen expression. Transfected genes that did not result in increased levels of β_2-microglobulin were assumed not to encode class I gene products. However, not all of the class I genes identified by Southern blot analysis of the murine genome were positive in this assay (48). It is possible that one of the genes which does not associate with β_2-microglobulin codes for the unique product precipitated by 48.21.

One possibility which cannot be excluded is that the recombinant analysis located a regulatory gene between *S* and *D* which controls the expression of a cell surface antigen encoded elsewhere. In such a model, the surface antigen would be involved in the regulation of the response to TNP-Ficoll and be recognized by the monoclonal antibody 48-21.7. It should be noted, however, that a regulatory gene of this nature would have to exert its control on allelic structural genes in a haplotype-specific manner. While this is possible, no such mechanism has been reported (49).

The relationship of the two loci required for the response to TNP-Ficoll remains unclear. Blocking experiments indicated that cell surface expression of high responder alleles of both loci are mandatory for optimum response. Immunoprecipitation analysis suggested that there was no physical association

between the I-A gene product and the product of the right-hand locus because antibodies specific for each of these loci precipitated distinct products (data not shown). The functional role of each of these molecules is unclear. It is possible that the I-A molecule acts as a traditional Ia antigen recognition structure together with some other type of receptor, membrane-bound enzyme, or another surface determinant encoded between the *H-2S* and *D* regions. There are several instances of interactions between genes of the MHC. In addition to the complementation of genes in the *I-A* and *I-E* regions for an Ia molecule (50), there are examples of interactions between the *H-2D* and *Qa* and *Tla* regions leading to altered expression of one of the molecules (29, 30, 51). Since an immune response function is associated with the 48.21.7 molecule, the TNP-Ficoll system may prove to be a useful model for studying these interactions. It will be particularly interesting to determine whether the 48.21 locus corresponds to any of the cloned genes clustering in this region for which no function or product has yet been shown. Further characterization of the 48.21.7 product and its locus is in progress.

Summary

The primary in vitro antibody response to the type 2 antigen, trinitrophenyl (TNP)-Ficoll, is controlled by two complementing loci in the *H-2* region of the mouse major histocompatibility complex (MHC). High responder alleles at both loci are necessary for a high responder phenotype. Previous studies mapped one locus of control to the *I-A* subregion. In this report we demonstrate by recombinant analysis that the second locus of control is located between the *H-2S* and *D* regions. A comparison of responses in the B10.BAR6, B10.BAR10, and B10.BAR11 strains defined a locus controlling the response to TNP-Ficoll in a single haplotype, bounded on the left by the crossover event in the B10.BAR10 and on the right by the crossover event in the B10.BAR6 strain. A monoclonal antibody directed against this right-hand region of control has been produced (48.21.7) that blocks the response to TNP-Ficoll at the level of the antigen-presenting cell. The monoclonal antibody 48-21.7 is specific for the high responder *b* allele at the right-hand locus and did not inhibit responses to other protein antigens tested. The immune response to TNP-Ficoll was not inhibited by monoclonal antibodies that react with $H\text{-}2D^b$ or *Qa-2* specificities, suggesting that the TNP-Ficoll response is controlled by a unique locus located between *H-2S* and *D*. Finally, 48-21.7 recognizes and precipitates a unique product of ~40,000 mol wt that is distinct from the *H-2D* region product recognized by the monoclonal antibody B22/249.

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