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*

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For some years there has been intense interest in the problem of cellular expression of immune response (*Ir*)[1] genes. Initial experiments suggested that T cells alone were responsible for *Ir* manifestations since, for example, mice which were low responders to poly-L-(Tyr,Glu)-poly-D,L-Ala--poly L-Lys [(TG)-A--L] could make antibodies to this antigen coupled to methylated bovine serum albumin (1) and guinea pigs nonresponsive to the dinitrophenyl (DNP) hapten coupled to poly-L Lys could make antibodies to DNP coupled to other carriers (2).

The idea of T-cell expression of *Ir* genes was strengthened by later experiments which showed that T cells from high responder strains could divide in response to the appropriate antigen, whereas T cells from low responder strains could not (3–5). In an elegant experiment, Bechtol et al. (6) showed that low responder B cells could make antibody to (TG)-A--L in tetraparental low responder ↔ high responder mice. This result again suggested that *Ir* genes were not expressed in B cells. The role of macrophages (Mϕs), however, was not studied in any of these experiments.

More recently, however, *Ir* genes have been found to be associated with B cells and/or Mϕs. In the guinea pig, F_1, low responder × high responder T cells proliferated when cultured with high responder Mϕs pulsed with antigen, but not with low responder Mϕs (7, 8). Similarly, Katz et al. (9) have shown in the mouse that F_1, high responder × low responder T cells would help antibody responses of B cells from high responder mice, but not low responder mice to DNP-poly L-Glu, L-Lys, L-Tyr. Our work has shown that the *Ir* gene controlling cross-reaction between sheep erythrocytes (SRBC) and burro erythrocytes (BRBC) at the helper T-cell level is expressed at least by the B cell in vitro (10, 11).

The work of several laboratories (12–18) has suggested that at least three types of low responder animals can exist. The first type, exemplified by the response of mice of the *H-*

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[1] *Abbreviations used in this paper:* B6AF$_1$, C57BL/6 × A F_1; B10, C57BL/10; BRBC, burro erythrocytes; BSS, balanced salt solution; CFA, complete Freund's adjuvant; DNP, dinitrophenyl(ated); GZ, beta-galactosidase; HRBC, horse erythrocytes; *Ir*, immune response; KLH, keyhole limpet hemocyanin; LPS, bacterial lipopolysaccharide; MHC, major histocompatibility complex; Mϕ, macrophage; PFC, plaque-forming cell; SRBC, sheep erythrocytes; (TG)-A--L, poly-L-(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys; TNP, trinitrophenyl(ated).

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2^k haplotype to (TG)-A--L, is unable to respond because it has no functional B cells for this antigen, even though helper T cells are present (13–15). The second, exemplified by the response of mice of the *H-2*^{f} haplotype to (TG)-A--L, is unable to respond because it lacks helper T cells even though B cells responsive to this antigen are present (12). Finally, animals may lack both T cells and B cells responsive to a particular antigen, exemplified by the response of some *H-2*^{s} mice to (TG)-A--L and to poly(Phe,Glu)-poly-L-ala-poly-L-Lys (16–18).

In almost all cases studied, response or lack of response to a particular antigen maps in or close to the major histocompatibility complex (MHC) of the species in question (15, 19, 20). This is true even when separate genetic controls for B and T cells have been shown to exist, though there may be some non-MHC-encoded influence on at least B-cell responsiveness (18, 20).

Given the continuing controversy over the expression of *Ir* genes in T cells, B cells, or Mϕs, we decided to dissect the response in vitro, where purified cell populations can more easily be separated and titrated together than in vivo. Direct plaque-forming cell responses to trinitrophenylated (TNP) (TG)-A--L in mouse spleen cell cultures were studied. These proved to have the same strain distribution of response as IgG responses to (TG)-A--L in vivo (19). When high responder × low responder F_1 cells were titrated with various combinations of B cells and Mϕs of either the parental or F_1 *H-2* type, high responsiveness required the presence of at least high responder B cells, and, in the one case studied, high responder Mϕs in the cultures, indicating the expression of *Ir* genes in both B cells and Mϕs.

Materials and Methods

Mice. B10.A × C57BL10/Sn (B10) F_1, B10.M × B10 F_1, B10.M × B10.A F_1, B10.M, B10.A (4R), and CBA/J × C3H.SW/Sn F_1 were bred in our vivarium. Breeding mice for the B10.M, B10.A(4R), C3H.SW/Sn and B10.S strains were kindly provided us by Doctors M. Cherry and J. Stimpfling. All other mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Cultures. Mouse spleen and lymph node cells were cultured by the methods of Mishell and Dutton (21), with some modifications (11).

Antigens. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, Calif., and after dissolving in saline, was centrifuged at 78,000 *g* for 2 h (22). The pellet was then redissolved in saline and sterilized before storage. (TG)-A--L was purchased from Miles Laboratories Inc., Elkhart Ind. During these experiments two different lots were used (numbers MC3 and MC6), both of which had been previously tested by Miles and shown to give high titres of antiserum in responder, C3H.SW, and low titres of antisera in low responder, C3H/HeJ or C3H/DiSn, mice. The two lots proved indistinguishable in our hands.

TNP_{727}-KLH, assuming a mol wt of 8×10^6 for KLH, and TNP-β-galactosidase (TNP_{17}-GZ) were prepared by the method of Rittenberg and Pratt (23). TNP-(TG)-A--L was prepared similarly with the following exceptions. 50 mg of (TG)-A--L and 7 mg of trinitrobenzene sulfonic acid were mixed in 3 ml of cacodylate buffer (pH 6.9). After 30 sec, the reaction was stopped by the addition of excess glycylglycine. Samples having 2.6 and 3.2 TNP substitutions/100,000 daltons (TG)-A--L were used in these experiments. ^{125}I-TNP-(TG)-A--L was prepared by standard methods (24). 5 mg of TNP-(TG)-A--L was dissolved in 0.2 ml saline to which 0.1 ml 0.1 M sodium borate, pH 7.8, was added. To this, 7.5 μl ^{125}I-labeled sodium iodide was added. The mixture was vortexed and stood at room temperature for 1 min. 0.5 ml of 4 μM iodine chloride was then added, and the mixture was dialyzed extensively against saline followed by balanced salt solution (BSS).

TNP-*Escherichia coli* lipopolysaccharide (TNP-LPS) was prepared as described previously (25).

Immunizations. Mice were immunized to yield (TG)-A--L-specific T cells by injection of 100 μg (TG)-A--L in 40 μl complete Freund's adjuvant (CFA) in the base of the tail (26, 27). 7 days later, the periaortic and inguinal lymph nodes were removed and used as a source of T cells. The

spleens of mice injected i.p. with 20–50 μg of KLH in CFA were used as a source of KLH-primed T cells. The spleens of mice injected i.p. 7 days previously with 1 μg TNP-LPS were used in most cases as a source of TNP-primed B cells (28). Such cells were primed in C3H/HeJ and C3H.SW/Sn mice by i.p. injection of 100 μg TNP-GZ in CFA 4–8 wk before use. In vitro immunization of cultures with TNP-KLH was by addition of 0.1 μg/ml TNP-KLH or as described below.

Antigen-Pulsed Mϕs. Mϕs were pulsed with antigen by modifications of the method of Pierce et al. (29). Briefly, the peritoneal cavities of normal mice were washed with ice-cold BSS. These washings were then centrifuged, and the cells were resuspended to 2×10^6/ml in ice-cold BSS. The relevant antigen, TNP-(TG)-A--L or TNP-KLH, was added to a final concentration of 100 μg/ml, and the mixture was incubated on ice for 1 h. The cells were than washed exhaustively with ice-cold BSS and counted before use in vitro. Experiments in which Mϕs were pulsed with ^{125}I-TNP-(TG)-A--L showed that 10^6 Mϕs bound ≅150 ng of antigen. Of this, ≅80% of the antigen was released during overnight culture. This was true for Mϕs from both high responder and low responder strains. Although peritoneal washings clearly contain a heterogeneous population of cells, including lymphocytes and Mϕs (30), the antigen-presenting cells will be referred to as Mϕs throughout the rest of the paper since in our hands the functional cells have the following properties, all of which are characteristic of Mϕs. They adhere to nylon fiber and Sephadex G-10. They are present in plastic adherent cells, >99% of which phagocytose latex particles. They are irradiation- and anti-T serum-plus-complement-resistant. Recent studies by others have, however, suggested that Mϕs themselves may be heterogeneous, both in the Ia antigens they bear (31, 32), and in their biological and biochemical properties (32, 33). Yamashita and Shevach (32) have reported that it is the Ia-positive subpopulation of Mϕs which is most efficient at antigen presentation in their experiments. We have not characterized the active subpopulation in our experiments.

T and B Cells. (TG)-A--L-specific T cells were prepared from the periaortic and inguinal lymph nodes of immunized mice (26, 27), KLH-specific T cells were isolated from the spleens of KLH-immunized mice. In both cases, the cells were passed through nylon fiber columns (11, 34, 35) to remove B cells, Mϕs, and other nylon fiber-adherent cells before use in vitro. B cells were isolated from spleen cell suspensions from TNP-primed mice by treatment of the cells with anti-T serum and complement (36). In some experiments it was also necessary to remove Mϕs from the B-cell populations. In these cases the B cells were Mϕ-depleted by passage through Sephadex G-10 columns (37) before treatment with anti-T serum and complement.

Direct Plaque-Forming Cell (PFC) Assay. After 4 days of culture, two or three identical culture wells were pooled and assayed in duplicate for direct anti-TNP PFC using the slide modification (21) of the Jerne hemolytic plaque assay. Parallel determinations were made using TNP-horse erythrocytes (TNP-HRBC) and HRBC, and the difference was recorded as the number of anti-TNP PFC. For these assays, lightly conjugated TNP-HRBC were prepared by the method of Rittenberg and Pratt (23), as modified by Kettman and Dutton (38). HRBC from a single animal were obtained from the Colorado Serum Co., Denver, Colo.

Assay of Helper T-Cell Activity. Helper T-cell activity was titrated as previously described (28). Culture wells were set up containing 3×10^6 TNP-primed B cells with or without Mϕs from the appropriate strain of mouse. For TNP-(TG)-A--L responses, 10^5–2×10^5 TNP-(TG)-A--L-pulsed Mϕs were added to each culture. For TNP-KLH responses, either 10^5–2×10^5 TNP-KLH-pulsed Mϕs were added to each culture, or the culture medium was supplemented with TNP-KLH to a final concentration of 0.1 μg/ml. Varying numbers of T cells primed to the appropriate antigen were then added to the cultures. A plot of anti-TNP PFC/culture vs. the number of helper cells added yielded a titration with an initially linear slope. The least squares line was fitted to the initial points, and the slope of the line was taken as the activity of the helper population (Fig. 1).

Results

Conditions for in Vitro Anti-TNP-(TG)-A--L Responses. Conditions were established under which we could observe anti-TNP-(TG)-A--L responses in vitro. In preliminary experiments we found that the conditions which we had previously used to study anti-TNP-KLH responses (28) were not sufficient to generate in vitro anti-TNP-(TG)-A--L responses. A number of alterations were required, as described below.

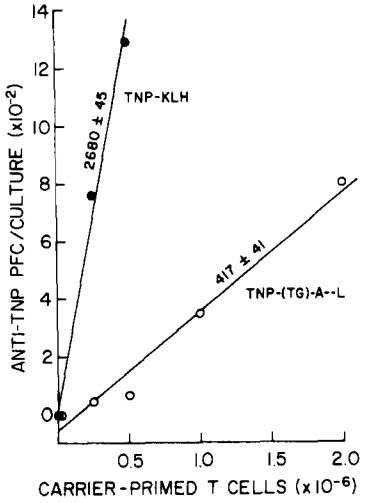

FIG. 1. Titration of (TG)-A--L-specific and KLH-specific helper T cells. Three B6AF$_1$ mice were primed with (TG)-A--L, and two with KLH. Seven days later, T cells were prepared from these mice and titrated for their helper activity in anti-TNP-(TG)-A--L responses (○) and anti-TNP-KLH responses (●), respectively, as described in Materials and Methods. The number of anti-TNP PFC/culture on day 4 is plotted vs. the number of (TG)-A--L-primed or KLH-primed T cells/culture. The initial slope ± SE of each titration line is also shown.

T cells isolated from the spleens of mice immunized i.p. with (TG)-A--L in CFA were not effective as helper cells in this response. This problem was solved by using T cells isolated from the periaortic and inguinal lymph nodes of mice immunized in the base of the tail with (TG)-A--L in CFA as described in Materials and Methods, and by others (26, 27). Such preparations were rich in (TG)-A--L-specific helper T cells.

We were unsuccessful in obtaining responses to TNP-(TG)-A--L when the antigen was added in soluble form to our cultures. This has been a common result when using soluble, relatively small antigens in our laboratory (39). This problem was solved by adding the antigen to cultures bound to peritoneal Mϕs (Materials and Methods).

As had been our previous experience with TNP-protein antigens in vitro (28), a vigorous anti-TNP-(TG)-A--L response in vitro required the use of TNP-primed B cells. These were prepared from the spleens of mice primed either with TNP-LPS or, in the case of mice of the C3H background, with TNP-GZ in CFA.

After these conditions had been satisfied, good anti-TNP-(TG)-A--L responses occurred in our cultures. For example, in Fig. 1 the anti-TNP-(TG)-A--L response of C57BL/6 × A/J (B6AF$_1$) B cells and Mϕs is plotted as a function of the number of (TG)-A--L-primed B6AF$_1$ T cells added to the cultures. Anti-TNP PFC numbers rose linearly as the numbers of (TG)-A--L-primed T cells in cultures were increased. In a control experiment, these same B cells and Mϕs were shown to respond well to TNP-KLH in the presence of increasing numbers of KLH-primed B6AF$_1$ T cells. Other experiments, not shown here, demonstrated that (TG)-A--L priming of the T cells was required, and that TNP-

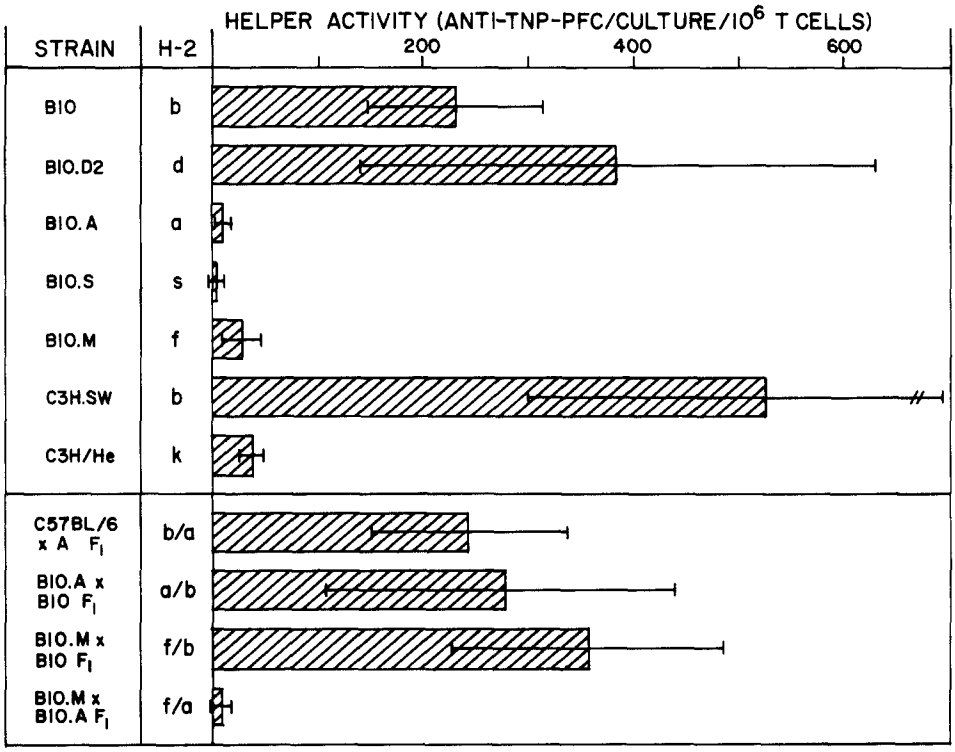

FIG. 2. Strain distribution of response to TNP-(TG)-A--L. T cells from different strains of mice were titrated for their helper activity in anti-TNP-(TG)-A--L responses as described in Materials and Methods and in Fig. 1. Shown here are the results of 38 separate determinations in which each strain was tested at least three times. For each titration, the number of anti-TNP PFC/culture was plotted vs. the number of T cells added, and the initial slopes were determined by the least squares method. Slopes for all determinations with a given strain were averaged and are shown ± SEM.

(TG)-A--L had to be present in the cultures for anti-TNP-(TG)-A--L responses to occur.

Strain Distribution of in Vitro Responses to TNP-(TG)-A--L. The ability of cells from different strains of mice to respond to TNP-(TG)-A--L was measured by titrating the ability of (TG)-A--L-primed T cells from each strain to stimulate the direct PFC response to TNP-(TG)-A--L of TNP-primed B cells and Mϕs from the same strain. As a control, KLH-primed T cells were also prepared in each strain and tested for their ability to stimulate an anti-TNP-KLH response in the same B cell and Mϕ population. The averaged results of a number of experiments on the strains so far tested are shown in Fig. 2. All strains responded well to TNP-KLH (results not shown). By contrast, B10, B10.D2/nSn, and C3H.SW mice all responded well to TNP-(TG)-A--L, but B10.A, B10.S, B10.M, and C3H/He mice responded poorly, if at all. Of the F_1 mice tested, B10.A × B10 F_1, B6AF_1, and B10.M × B10 F_1 all gave large responses, whereas B10.M × B10.A F_1 mice did not respond. These results are in agreement with the reports of in vivo IgG responses to (TG)-A--L (19) and with some of the published reports of in vivo IgM responses to (TG)-A--L (20, 40, 41), and in vitro IgM responses to TNP-(TG)-A--L (42), with exceptions discussed later.

TABLE I
Mapping of Ir Gene(s) for Anti-TNP-(TG)-A--L Responses in Vitro

Strain	*H-2* subregions*									Anti-TNP-(TG)-A--L response (PFC/10^6 T cells)‡
	K	*I-A*	*I-B*	*I-J*	*I-E*	*I-C*	*S*	*G*	*D*	
B10.A(5R)	b	b	b	k	k	d	d	d	d	685 ± 351
B10.A(4R)	k	k	b	b	b	b	b	b	b	8 ± 7
B10.A(2R)	k	k	k	k	k	d	d	d	b	4 ± 5
B10.A	k	k	k	k	k	d	d	d	d	8 ± 6§
B10.D2	d	d	d	d	d	d	d	d	d	385 ± 246§
B10	b	b	b	b	b	b	b	b	b	228 ± 88§

* Haplotype data from references 50–52.
‡ Average ± SEM of between 3 and 6 separate determinations.
§ Data from Fig. 1.

Experiments were performed in B10 congenic mice with recombinant *H-2* haplotypes to allow preliminary mapping of the *Ir* gene(s) controlling the in vitro direct PFC response to TNP-(TG)-A--L. As shown in Table I, B10.A(5R) mice responded well to the antigen, but B10.A(4R) and B10.A (2R) responded poorly, suggesting that the gene(s) controlling this phenomenon map in the *K*, *I-A* end of the *H-2* complex. This location for genes controlling in vivo and in vitro responses to (TG)-A--L has already been well established by others (15, 19, 42).

Expression of the Ir Gene(s) in B Cells. Having shown that the in vitro response to TNP-(TG)-A--L was under the control of *Ir* gene(s), we wished to determine which cell type(s) were expressing the gene(s) in vitro: T cells, B cells, or Mϕs. Since these cell types cannot be taken from different unrelated mice and mixed in vitro without generating mixed lymphocyte reactions and complicating allogeneic effects, we designed these experiments along the lines we have previously described (10, 11, 43, 44). Thus, (TG)-A--L-primed T cells were obtained from F_1 mice, the cross between high responder and low responder parents. These T cells were then titrated for their ability to stimulate anti-TNP-(TG)-A--L responses of B cells and Mϕs obtained from congenic mice identical at *H-2* with either the high responder or low responder parent. TNP-(TG)-A--L was added to the cultures bound to either high responder or low responder Mϕs. F_1 T cell and congenic B cell and Mϕ donors were also selected such that no anti-*Mls* activity (45) would be obtained. This protocol permitted cultures to be set up with cells from mice differing at *H-2*, and it eliminated undesirable allogeneic effects since the F_1 T cells were incapable of recognizing the *H-2* or *Mls* antigens of the B cells and Mϕs. We hoped that this protocol would determine whether high responder F_1 T cells were sufficient for a high anti-TNP-(TG)-A--L response, or whether high responder B cells and/or Mϕs were also required.

Three examples of this type of experiment are shown in Fig. 3. The results in Fig. 3 a show that when (TG)-A--L-immunized B6AF_1 (*H-2*^{b} × *H-2*^{a}) T cells were titrated for their ability to help anti-TNP-(TG)-A--L responses of B10 (*H-2*^{b}, high responder) or B10.A(2R) (*H-2*^{h2}, low responder) cells, high responses were obtained with B10 cells and low responses with B10.A(2R) cells, irrespective of the type of Mϕ bearing antigen in the cultures. Identical experiments

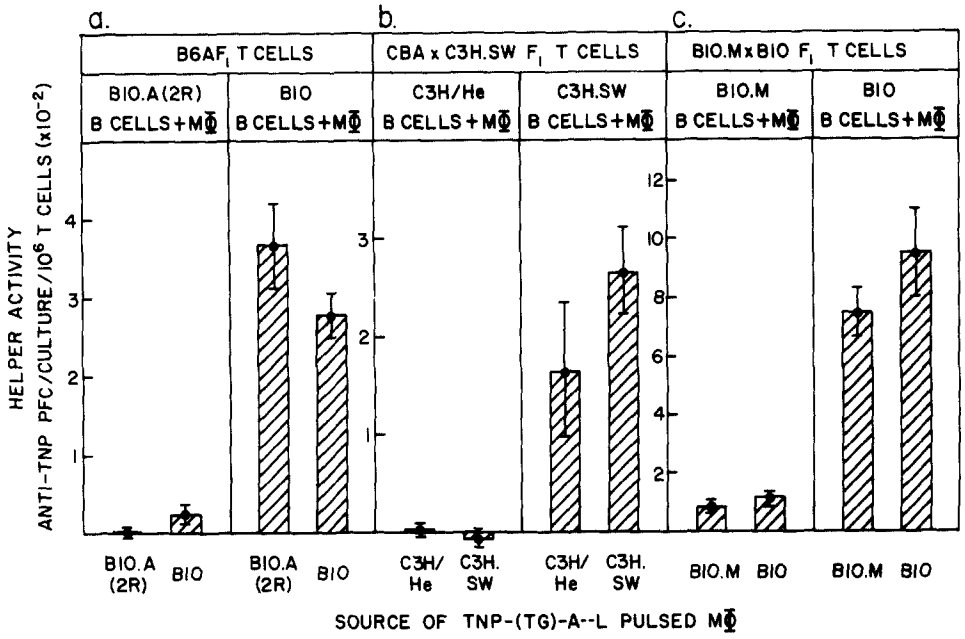

FIG. 3. Expression of Ir genes in B cells. F_1, high responder × low responder mice were primed with (TG)-A--L. Their T cells were subsequently titrated for helper activity in anti-TNP-(TG)-A--L responses of B cells and Mϕs from high or low responder mice congenic with the parents of F_1 at *H-2*. TNP-(TG)-A--L was added to cultures bound to high or low responder Mϕ. Slopes ± SE of titrations for different T-cell, B-cell and Mϕ, and antigen-bound Mϕ preparations are shown, obtained in representative experiments. Each experiment was performed three times. Results are shown for three different strain combinations. (a) B6AF$_1$ T cells, B10.A(2R) low responder, and B10 high responder B cells and Mϕs; (b) CBA × C3H.SW F_1 T cells, C3H/He low responder, and C3H.SW high responder B cells and Mϕs; (c) B10.M × B10 F_1 T cells, B10.M low responder, and B10 high responder B cells and Mϕs.

were performed with similar results using CBA/J × C3H.SW (*H-2*^{k} × *H-2*^{b}) F_1 (TG)-A--L-primed T cells and B cells and Mϕs from C3H/HeJ (*H-2*^{k}, low responder) or C3H.SW (*H-2*^{b}, high responder) mice (Fig. 3b). Another set of experiments was performed with B10.M × B10 F_1 (*H-2*^{f} × *H-2*^{b}) (TG)-A--L-primed T cells and B cells and Mϕs from B10.M (*H-2*^{f}, low responder) or B10 (*H-2*^{b}, high responder) mice, again with qualitatively similar results (Fig. 3c). It should be noted, however, that the anti-TNP-(TG)-A--L responses of B10 B cells and Mϕs, when stimulated by B10.M × B10 F_1 T cells, were the highest of all the strain combinations tested, and that the responses of B10.M B cells and Mϕs, when stimulated by the same T cells, were much smaller than these, but appreciable by comparison with other strain combinations. Each of these experiments has been performed three times with similar results.

Thus, in the three cases examined, the presence of high responder F_1 T cells was not a sufficient condition for high response. In each case, cultures also had to contain high responder B cells for good anti-TNP-(TG)-A--L responses to occur, indicating the expression of *Ir* genes in B cells. We were tempted to conclude from our experiments that the *Ir*-type of the antigen-presenting Mϕ

was irrelevant in the response. Some subsequent control experiments using ^{125}I-TNP-(TG)-A--L, however, indicated that this conclusion was not justified. In these experiments both high and low responder Mϕs were shown to take up approximately the same amount of TNP-(TG)-A--L during our pulsing procedure. More importantly, both types released $\cong$80% of this bound antigen within 24 h of culture. Thus, in the experiments shown in Fig. 3, there was a possibility of antigen-transfer from the original antigen-bearing Mϕs to those introduced with the B-cell preparation. The high responses obtained when F_1 T cells were cultured with high responder B cells and Mϕs and antigen-pulsed low responder Mϕs might have been due to antigen-transfer to the high responder Mϕs. The problem of *Ir*-gene expression in Mϕs in our cultures was, therefore, still unanswered. This issue was addressed in a further set of experiments described in the following section.

Two types of controls were performed for these mixing experiments. First, F_1 (TG)-A--L-primed T cells were titrated into cultures containing splenic B cells and Mϕs from one strain, and peritoneal Mϕs from the other strain, in the absence of antigen. TNP PFC/culture/10^6 T cells were always <2 under such circumstances, suggesting that no nonspecific stimulation of anti-TNP PFC responses was resulting from the mixing of peritoneal cells from one strain with B cells and Mϕs from the other, in the presence of T cells. In other control experiments, low responder B cells were shown to respond to TNP coupled to unrelated antigens by testing their response to TNP-KLH bound to high responder or low responder Mϕs in the presence of F_1 KLH-primed T cells (results not shown).

Expression of Ir Gene(s) in B Cells and Mϕ. To discover whether *Ir* genes were being expressed in Mϕs as well as B cells in our cultures, splenic B cells had to be depleted of Mϕs by passage over Sephadex G-10 columns. Such a maneuver prevented possible antigen transfer from the Mϕs on which it was added to cultures to Mϕs in our splenic B-cell populations. Thus B10.A or B10 B cells were depleted of Mϕs, TNP-(TG)-A--L was added to cultures bound to either B10 or B10.A Mϕs, and $B6AF_1$ (TG)-A--L-primed T cells were titrated into the cultures. The results of a typical experiment of three are shown in Fig. 4 a. As in the previous experiments, B10.A B cells did not respond to TNP-(TG)-A--L in the presence of $B6AF_1$ (TG)-A--L-primed T cells whether the antigen was added to cultures bound to B10.A or B10 Mϕs. B10 B cells responded to TNP-(TG)-A--L in the presence of helper T cells if the antigen was added to cultures on the surface of B10 Mϕs but, unlike the previous experiment, not if antigen was added on the surface of B10.A Mϕs. These results suggested that *Ir* genes were being expressed in vitro by both B cells and Mϕs. Again, in control experiments both the B10 and B10.A Mϕs and B cells were shown to be functional in anti-TNP-KLH responses in the presence of KLH-primed F_1 T cells.

These results could also be explained by a theory in which *Ir* genes are expressed only in B cells or Mϕs in vitro, but *H-2* compatibility is required for good B cell-Mϕ cooperation. Thus, *Ir* genes might be expressed in Mϕs only, for example, but B10 Mϕs would be unable to cooperate with B10.A B cells because the two are histoincompatible. To eliminate this explanation, experiments were

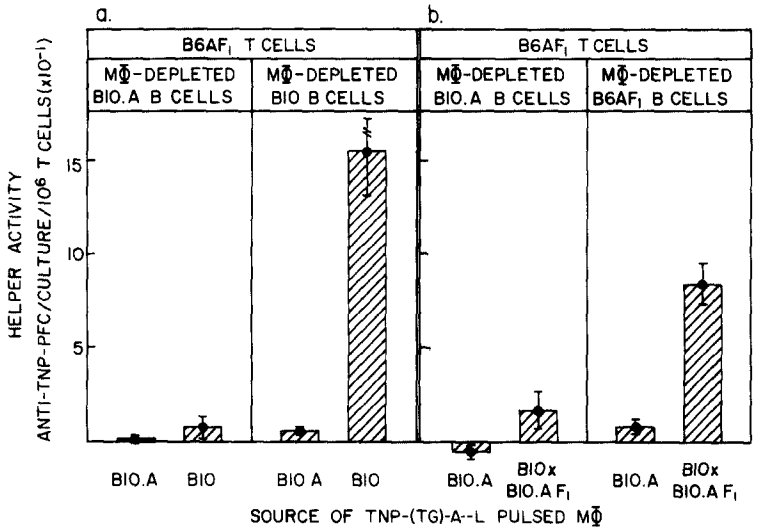

FIG. 4. Expression of Ir genes in B cells and Mϕs. (a) B6AF$_1$ mice were primed with (TG)-A--L and their T cells were subsequently titrated for helper activity in anti-TNP-(TG)-A--L responses of Mϕ-depleted B cells from B10.A, low responder, or B10, high responder animals. Antigen was added to cultures bound to B10.A or B10 Mϕs. The initial slopes ± SE obtained in a representative experiment of 3 are shown. (b) As in (a), except that Mϕ-depleted B cells were obtained from B10.A and B6AF$_1$ animals and antigen-pulsed Mϕ were obtained from B10.A and B10 × B10.A F$_1$ animals.

performed in which Sephadex G-10-passed B6AF$_1$ or B10.A B cells were tested for their response to TNP-(TG)-A--L bound to B10.A or B10 × B10.A F$_1$ Mϕs in the presence of B6AF$_1$ (TG)-A--L-primed T cells. In this experiment, B cells and Mϕs were always at least semihistocompatible, thus, *H-2*-controlled restrictions on B cell-Mϕ interactions should not occur. The results of one such experiment is shown in Fig. 4b. B10.A B cells did not respond to TNP-(TG)-A--L , even if the antigen was added to cultures bound to B10 × B10.A F$_1$, semihistocompatible, responder Mϕs. Conversely, the response of B6AF$_1$ B cells to TNP-(TG)-A--L in the presence of F$_1$ helper T cells ocurred only if antigen was added to the cultures bound to B10 × B10.A F$_1$ Mϕs, not if bound to B10.A Mϕs. These results were repeated in two other experiments. These results show again that *Ir* gene(s) controlling (TG)-A--L responses are being expressed by both B cells and Mϕs in vitro, and that our results cannot be explained by lack of interaction between histoincompatible B cells and Mϕs.

Discussion

The problem of cellular expression of *Ir* genes in antibody responses seems to be particularly difficult to solve, since at one time or another B cells, Mϕs, and T cells have all been implicated. Proliferation experiments in guinea pigs and mice, for example, have shown that at least Mϕs, and possibly also T cells, may express the genes (5, 7, 8). Other experiments have shown that B cells may also be involved (9–11). The problem is not simplified if the experiments considered are confined to those involving only one antigen, (TG)-A--L. McDevitt et al. (4), Bechtol et al. (6) have shown that *H-2*^{k}-bearing B cells in *H-2*^{k} ↔ *H-2*^{b}

tetraparental mice are not defective in their ability to respond to this antigen, although T cells and/or Mϕs from mice of the *H-2*^{k} haplotype are apparently nonresponsive (3). On the other hand, the experiments of Munro and Taussig (12), Litchenberg et al. (14), Erb and Feldmann (13), and Howie and Feldmann (46) suggest that *H-2*^{k} B cells are defective and *H-2*^{k} T cells are functional in anti-(TG)-A--L responses.

According to Munro and Taussig (12), Lichtenberg et al. (14), Mozes et al. (16), and Munro et al. (18), however, in other strains of mice the defect in response to (TG)-A--L may lie in either the T cells alone (B10.M, A.CA, *H-2*^{f}; A.SW, *H-2*^{s}), or in the T cells and B cells (S.JL: *H-2*^{s}). The role of the Mϕ was not studied in these experiments, but it has been suggested by others that so-called T-cell-defective mice did in fact contain deficient Mϕs.

Since it is very difficult to deplete mice of Mϕs, it seemed reasonable to us to examine the possible expression of *Ir* genes for (TG)-A--L by B cells and Mϕ in vitro. Thus, we established conditions in vitro under which antibody responses to TNP coupled to (TG)-A--L could be obtained. Our direct PFC responses were secondary since both B cells and T cells were primed to antigen. When the response of cells from different strains of mice to TNP-(TG)-A--L was measured, the pattern of response was as predicted from the in vivo distribution of IgG responses to (TG)-A--L (19) and a recently published survey of IgM primary responses to TNP-(TG)-A--L in vitro (42). The one exception to this was our observation that B10.D2 mice were high responders to TNP-(TG)-A--L, whereas in vivo results predict that they should be intermediate. There is some controversey in the literature over *Ir* restriction of IgM responses to (TG)-A--L (20, 40–42). Results seem to depend on the antigen and conditions used. Our system, however, detects *Ir*-controlled differences in direct PFC responses.

We were somewhat surprised to find that mice of the *H-2*^{a} and *H-2*^{k} haplotypes were low responders to TNP-(TG)-A--L, since the experiments of Howie and Feldmann (46) show that they should be high responders. Singer et al. (42), who also show that *H-2*^{a} and *H-2*^{k} mice are nonresponders to TNP-(TG)-A--L, have suggested that this anomalous result of Howie and Feldmann (46) results from their methods of preparing antigen. We were also disappointed to note that B10.M × B10.A F_1 mice were low responders to TNP-(TG)-A--L in our hands. Munro and Taussig (12) and Munro et al. (18) originally showed that gene complementation occurred in this F_1 combination, a result which is very intriguing. In subsequent experiments, McDevitt (41) and Munro and Taussig (47) have been unable to repeat the original finding, in agreement with our result.

We concluded, however, that our in vitro IgM secondary responses were measuring the presence of an *Ir* gene(s) with a strain distribution identical to that identified by others in vivo and in vitro. Moreover, this gene(s) mapped in the same part of the *H-2* complex as gene(s) controlling other (TG)-A--L responses, viz *K*, *I-A*.

Having established this, we set up experiments along the lines originally published by Katz et al. (9) and Shevach and Rosenthal (7), and more recently ourselves (10, 11). (TG)-A--L primed T cells were obtained from F_1 mice, the cross between high and low responder parents. These T cells were titrated for their ability to help anti-TNP-(TG)-A--L responses of B cells and Mϕs of either

parental *H-2* type. Three different strain combinations were studied. In every case, low responder B cells (B10.A(2R), $H\text{-}2^{h2}$; C3H/HeJ, $H\text{-}2^{k}$; B10.M, $H\text{-}2^{f}$) were unable to respond in the presence of active T cells and Mϕs of the high responder type. We concluded from these experiments that *Ir* genes were being expressed at least by B cells in all three low responder strains studied here.

We wished to test for the expression of *Ir* genes by Mϕs in antibody responses. To do this, Mϕs were removed from our B-cell populations to prevent antigen transfer, and F_1 cells were titrated into cultures containing B10.A ($H\text{-}2^{a}$, low responder) or B10 ($H\text{-}2^{b}$, high responder) B cells. Antigen was added on B10.A or B10 Mϕs. B10.A B cells did not respond to TNP-(TG)-A--L, regardless of the Mϕ type in vitro. B10 B cells responded only when B10 Mϕs were added. Using F_1 Mϕs, we were able to show that the lack of response of B10.A B cells was not due to *H-2* differences between the B cells and Mϕs.

We therefore concluded that B10.A mice were low responders to TNP-(TG)-A--L because they lacked both functional B cells and functional Mϕs for this antigen. Of the other strains studied, C3H/HeJ ($H\text{-}2^{k}$), B10.A(2R), and B10.M ($H\text{-}2^{f}$) lacked at least functional B cells. Our finding that B10.M B cells are nonfunctional is in direct contradiction to the results of Munro and Taussig (12), who suggest that B10.M mice should contain functional B cells and nonresponsive T cells for (TG)-A--L. This is a contradiction we are at present unable to resolve except by pointing out that the conditions of our experiments are vastly different.

Although the antigen presenting cells in our cultures have been identified as Mϕs by several criteria (Materials and Methods), we have not characterized the subpopulation of Mϕs which are active in our experiments. Since it has been shown by other investigators that it is the Ia-positive subpopulation of Mϕs which are most active in antigen presentation (32), our future experiments will be designed to test whether it is the Ia-positive Mϕs which differ in their ability to present TNP-(TG)-A--L between low responder and high responder strains of mice.

None of the experiments described in this paper examine the question of *Ir* gene expression in T cells. Thus, all the low responder strains we have tested may also contain nonfunctional T cells for (TG)-A--L responses. Our future experiments will be designed to tackle this question.

The results which we present here are consistent with our previous findings (11) that the *Ir* gene which controls the ability of helper T cells to respond to a cross-reacting determinant on SRBC and BRBC is expressed at least at the level of the B cell.

A number of models have been proposed to explain how MHC-linked *Ir* genes can control the activity of helper T cells even though they are expressed in B cells and Mϕ (12, 48, 49). At present, we favor the associative or dual recognition hypothesis in which the specificity of helper T cells is determined by the simultaneous recognition of antigen and *Ir*-gene products on either the Mϕ or B-cell surface (49). Thus, B cells and Mϕs in high responder strains would possess, and in low responder strains would lack the appropriate I-region encoded molecule which could be recognized in association with the antigen. The attraction of this model is that it is consistent with a large body of evidence

in the literature (13, 29, 48, 49), including work from this laboratory (11, 43, 44) concerning the interaction of helper T cells with antigen.

Summary

Using lymph node T cells from poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys [(TG)-A--L]-primed animals and B cells from animals primed with trinitrophenylated (TNP) protein or lipopolysaccharide, we have obtained anti-TNP-(TG)-A--L direct plaque-forming responses in vitro. Response to this antigen was shown to be controlled by the *H-2* haplotype of the animal studied. The strain distribution of in vitro response was very similar to that previously reported by others for in vivo secondary IgG responses to (TG)-A--L.

We investigated the cell types expressing the *Ir* gene(s) for (TG)-A--L in our cultures. F_1, high responder × low responder mice were primed with (TG)-A--L. Their T cells were active in stimulating anti-TNP-(TG)-A--L responses of high responder but not low responder B cells and macrophages (Mϕ), even though both preparations of B cells and Mϕ were obtained from mice congenic at *H-2* with one of the parents of the F_1. For three low responder strains tested, of the $H\text{-}2^{h2}$, $H\text{-}2^{k}$, and $H\text{-}2^{f}$ haplotypes, the anti-TNP-(TG)-A--L response of low responder B cells and Mϕs in the presence of high responder, F_1 T cells could not be improved by the addition of high responder, antigen-bearing Mϕs to the cultures. In one strain of the $H\text{-}2^{a}$ haplotype, it was shown that neither the B cells nor Mϕs could be functional in anti-TNP-(TG)-A--L responses. Our results therefore suggested the *Ir* genes for anti-TNP-(TG)-A--L responses were expressed at least in B cells in all the low responder strains we studied, and, in mice of the $H\text{-}2^{a}$ haplotype, in Mϕs too.

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References

1. McDevitt, H. O. 1968. Genetic control of the antibody response. III. Qualitative and quantitative characterization of the antibody response to (TG)-A--L in CBA and C57 mice. *J. Immunol.* 100:485.
2. Green, I., W. E. Paul, and B. Benacerraf. 1966. The behavior of hapten-poly-L-lysine conjugates as complete antigens in genetic responder and as haptens in nonresponder guinea pigs. *J. Exp. Med.* 123:859.
3. Lonai, P., and H. O. McDevitt. 1974. Genetic control of the immune response. In vitro stimulation of lymphocytes by (TG)-A--L , (HG)-A--L and (Phe,G)-A--L. *J. Exp. Med.* 140:977.
4. McDevitt, H. O., K. B. Bechtol, G. J. Hammerling, P. Lonai, and T. L. Delovitch. 1974. *Ir* genes and antigen recognition. *In* The Immune System: Genes, Receptors, Signals. Third ICN-UCLA Symposium on Molecular Biology. E. E. Sercarz, A. R. Williamson, and C. F. Fox, editors. Academic Press Inc., New York. 597.
5. Schwartz, R. H., and W. E. Paul. 1976. T-lymphocyte-enriched murine peritoneal

exudate cells. II. Genetic control of antigen-induced T-lymphocyte proliferation. *J. Exp. Med.* **143**:529.

6. Bechtol, K. B., J. H. Freed, L. A. Herzenberg, and H. O. McDevitt. 1974. Genetic control of the antibody response to poly-L(Tyr,Glu)-poly-D,L-Ala--poly-L-Lys in C3H ↔ CWB tetraparental mice. *J. Exp. Med.* **140**:1660.
7. 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 regulation of genetic control of the immune response. *J. Exp. Med.* **138**:1213.
8. Rosenthal, A. S., M. A. Barcinski, and J. T. Blake. 1977. Determinant selection is a macrophage dependent immune response gene function. *Nature (Lond.).* **267**:156.
9. Katz, D. H., T. Hamaoka, M. E. Dorf, P. H. Maurer, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of the immune response (*Ir*) gene in the control of lymphocyte interactions in responses controlled by the gene. *J. Exp. Med.* **138**:734.
10. Kappler, J. W., and P. C. Marrack. 1978. Simultaneous recognition of carrier antigens and products of the *H-2* complex by helper T cells. *In* Immune System: Genetics and Regulation. E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press Inc., New York. 439.
11. Kappler, J. W., and P. C. Marrack. 1977. The role of *H-2* linked genes in helper T-cell function. I. In vitro expression in B cells of immune response genes controlling helper T-cell activity. *J. Exp. Med.* **146**:1748.
12. Munro, A. J., and M. J. Taussig. 1975. Two genes in the major histocompatibility complex control response. *Nature (Lond.).* **256**:103.
13. Erb, P., and M. Feldmann. 1975. The role of macrophages in the generation of T-helper cells. II. The genetic control of the macrophage-T-cell interaction for helper cell induction with soluble antigens. *J. Exp. Med.* **142**:460.
14. Lichtenberg, L., E. Mozes, G. M. Shearer, and M. Sela. 1974. The role of thymus cells in the immune response to poly (tyr,glu)-poly-D,L-ala--poly-lys as a function of the genetic constitution of the mouse strain. *Eur. J. Immunol.* **4**:430.
15. Taussig, M. J., A. J. Munro, R. Campbell, C. S. David, and N. A. Staines. 1975. Antigen-specific T-cell factor in cell cooperation. Mapping within the *I* region of the *H-2* complex and ability to cooperate across allogeneic barriers. *J. Exp. Med.* **142**:694.
16. Mozes, E., R. Isac, and M. J. Taussig. 1975. Antigen-specific T-cell factors in the genetic control of the immune response to poly(Tyr,Glu)-poly-DLAla--polyLys. *J. Exp. Med.* **141**:703.
17. Shearer, G. M., E. Mozes, and M. Sela. 1972. Contribution of different cell types to the genetic control of immune responses as a function of the chemical nature of the polymeric side chains (poly-L prolyl and poly-DL-alanyl) of synthetic immunogens. *J. Exp. Med.* **139**:1009.
18. Munro, A., M. Taussig, and J. Archer. 1977. I-region products and cell interactions: contribution of non *H-2* genes to acceptor and factor for (TG)-A--L. *In* Third *Ir* Gene Workshop. H. O. McDevitt, editor. Academic Press Inc., New York. In press.
19. McDevitt, H. O., B. D. Deak, D. C. Shreffler, J. Klein, J. H. Stimpfling, and G. D. Snell. 1972. Genetic control of the immune response. Mapping of the *Ir-1* locus *J. Exp. Med.* **135**:1259.
20. Smith, S. M., D. B. Ness, J. A. Talcott, and F. C. Grumet. 1977. Genetic control of IgM responses to (T,G)-A--L, *H-2* and *Ig-1* linkage. *Immunogenetics.* **4**:221.
21. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
22. Weigle, W. O. 1966. The induction of a hyporesponsive state to hemocyanin. *J. Immunol.* **96**:319.

23. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenol (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* 132:575.
24. Bale, W. F., R. W. Helkamp, T. P. Davis, M. J. Izzo, R. L. Goodland, M. A. Contreras, and I. L. Spar. 1966. High specific activity labelling of proteins with L^{131} by the iodine monochloride method. *Proc. Soc. Exp. Biol. Med.* 122:407.
25. Jacobs, D., and D. C. Morrison. 1974. Stimulation of a T-independent primary anti-hapten response *in vitro* by TNP-lipopolysaccharide (TNP-LPS). *J. Immunol.* 114:360.
26. Ruddle, N. H. 1974. Cytotoxicity reaction mediated by antigen activated rat and mouse lymphocytes. S. Cohen and R. T. McCluskey, editors. John Wiley & Sons, Inc., New York. 401.
27. Corradin, G., H. M. Etlinger, and J. M. Chiller. 1977. Lymphocyte specificity to protein antigens. I. Characterization of the antigen-induced *in vitro* T cell-dependent proliferative response with lymph node cells from primed mice. *J. Immunol.* 119:1048.
28. Marrack, P., and J. W. Kappler. 1976. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. II. Two helper cells distinguished by their antigen sensitivities. *J. Immunol.* 116:1373.
29. Pierce, C. W., J. A. Kapp, and B. Benacerraf. 1976. Regulation by the *H-2* gene complex of macrophage-lymphoid cell interactions in secondary antibody responses in vitro. *J. Exp. Med.* 144:371.
30. Goodman, J. W. 1964. On the origin of peritoneal fluid cells. *Blood.* 23:18.
31. Cowing, C., B. D. Schwartz, and H. B. Dickler. 1978. Macrophage Ia antigens. I. Macrophage populations differ in their expression of Ia antigens. *J. Immunol.* 120:378.
32. Yamashita, U., and E. M. Shevach. 1977. The expression of Ia antigens on immunocompetent cells in the guinea pig. II. Ia antigens on macrophages. *J. Immunol.* 119:1584.
33. Snyderman, R., M. C. Pike, D. G. Fischer, and H. S. Koren. 1977. Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. *J. Immunol.* 119:2060.
34. Julius, M. F., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
35. Greaves, M. F., and G. J. Brown. 1974. Purification of human T and B lymphocytes. *J. Immunol.* 112:420.
36. Kappler, J. W., and P. C. Marrack (Hunter). 1975. Functional heterogeneity among the T-derived lymphocytes of the mouse. III. Helper and suppressor T cells activated by concanavalin A. *Cell. Immunol.* 18:9.
37. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* 5:239.
38. Kettman, J. R., and R. W. Dutton. 1970. An *in vitro* primary response to 2,4,6-trinitrophenyl substituted erythrocytes: response against carrier and hapten. *J. Immunol.* 104:1558.
39. Hoffeld, J. T., P. Marrack, and J. W. Kappler. 1976. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. IV. Development of a model system demonstrating responsiveness of two T cell functions to HGG *in vitro*. *J. Immunol.* 117:1953.
40. Grumet, F. C. 1972. Genetic control of the immune response. A selective defect in immunologic (IgG) memory in nonresponder mice. *J. Exp. Med.* 135:110.
41. McDevitt, H. O. 1976. *In* The Role of Products of the Histocompatibility Gene Complex in Immune Responses. D. H. Katz and B. Benacerraf, editors. Academic

Press Inc., New York. 321.

42. Singer, A., H. B. Dickler, and R. J. Hodes. 1977. Cellular and genetic control of antibody responses in vitro. II. Ir gene control of primary IgM responses to trinitrophenyl conjugates of poly-L-(Tyr,Glu)-poly-D,L-Ala-poly-L-Lys and poly-L-(His,Glu)-poly-D,L-Ala-poly-L-Lys. *J. Exp. Med.* **146**:1096.
43. Kappler, J. W., and P. C. Marrack. 1976. Helper T cells recognize antigen and macrophage surface components simultaneously. *Nature (Lond.)*. **262**:797.
44. Swierkosz, J. E., K. Rock, P. Marrack, and J. W. Kappler. 1978. The role of *H-2*-linked genes in helper T-cell function. II. Isolation on antigen-pulsed macrophages of two separate populations of F_1 helper T cells each specific for antigen and one set of parental *H-2* products. *J. Exp. Med.* **147**:554.
45. Festenstein, H. 1973. Immunogenetic and biological aspects of *in vitro* lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* **15**:62.
46. Howie, S., and M. Feldmann. 1977. *In vitro* studies of *H-2*-linked unresponsiveness to synthetic polypeptides. II. Production of an antigen-specific T helper cell factor to (TG)-A--L. *Eur. J. Immunol.* **7**:417.
47. Munro, A. J., and M. J. Taussig. 1977. Complementation of immune response genes for (T,G)-A--L. *Nature (Lond.)*. **269**:355.
48. Katz, D. H., and B. Benacerraf. 1976. Genetic control of lymphocyte interactions and differentiation. *In* The Role of Products of Histocompatibility Gene Complex in Immune Response. D. H. Katz and B. Benacerraf, editors. Academic Press Inc., New York. 355.
49. Paul, W. E., and B. Benacerraf. 1977. Functional specificity of thymus-dependent lymphocytes. *Science (Wash. D. C.)*. **195**:1293.
50. David, C. S. 1976. Serologic and genetic aspects of murine Ia antigens. *Transplant. Rev.* **30**:299.
51. Murphy, D. B., L. A. Herzenberg, K. Okumura, L. A. Herzenberg, and H. O. McDevitt. 1976. A new *I* subregion (*I-J*) marked by a locus (*Ia-4*) controlling surface determinants on suppressor T lymphocytes. *J. Exp. Med.* **144**:699.
52. Shreffler, D. C., C. S. David, S. E. Cullen, J. A. Frelinger, and J. E. Niederhuber. 1978. Serological and functional evidence for further subdivision of the *I* regions of the H-2 gene complex. *Cold Spring Harbor Symp. Quant. Biol.* **41**:477.