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*

By JOHN W. KAPPLER and PHILIPPA MARRACK‡

(From Department of Microbiology, Division of Immunology, Cancer Center, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642)

The role of the major histocompatibility complex (MHC)[1] in the control of specific immune responsiveness has been a controversial issue among immunologists for the past 10 years. Particularly enigmatic has been the mode of action of immune response (*Ir*) genes which, in the mouse, have been generally mapped within the *I* region of the *H-2* complex. The initial findings that these genes apparently controlled the response of T cells to specific T-dependent antigens led to the suggestion that somehow the products of these genes were involved in the structure of the T-cell receptor for antigen (reviewed in references 1, 2). However, more recent experiments, initiated by the work of Katz and co-workers (3–5) studying T-cell/B-cell cooperation, and Rosenthal and Shevach (6, 7) studying macrophage (MΦ)/T-cell cooperation, have led to the conclusion that T-cell interactions with antigen can be controlled by MHC genes expressed in cells other than T cells, namely in B cells and/or MΦ.

The control of helper T-cell function by MHC genes has been studied for the most part in vivo under conditions where it has been difficult to precisely determine which cell types express the gene function. Part of the difficulty of studying these phenomena in vitro has been that the most widely used antigens the responses to which are under *Ir*-gene control have not been particularly good immunogens in vitro.

In this paper we report the existence of an *Ir* gene(s) controlling the response of helper T cells to sheep erythrocytes (SRBC), an antigen widely used in in vitro systems. The *Ir*-gene control was detected while studying the cross-reaction of SRBC-primed helper T cells with burro erythrocytes (BRBC) (8, 9). The extent of cross-reaction was found to vary among mouse strains according to *H-2* haplotype. In experiments in which T and B cells and MΦ of various *H-2* haplotypes were combined in vitro, we demonstrated the expression of the *Ir*

* Supported by U.S. Public Health Service research grants AI-11558 and CA-11198 and American Cancer Society research grant IM-49.

‡ Recipient of an Established Investigatorship from the American Heart Association.

Preliminary results of these studies were reported at the ICN-UCLA Symposia on Molecular and Cellular Biology: "Regulation of the Immune System", Park City, Utah, March 1977 (11).

[1] *Abbreviations used in the paper:* B10, C57BL/10; BRBC, burro erythrocytes; BSS, balanced salt solution; FCS, fetal calf serum; HRBC, horse erythrocytes; *Ir*, immune response; KLH, keyhole limpet hemacyanin; MΦ, macrophage; MHC, major histocompatibility complex; PFC, plaque-forming cells; SRBC, sheep erythrocytes; (T,G)AL, poly-L-(Try,Glu)-Poly-D,L-Ala-poly-L-Lys; TNP, trinitrophenyl(ated).

gene(s) in B cells, although expression in MΦ and in T cells was not excluded as a possibility.

We interpret these and our previous findings (10, 11) to support the view that MHC gene products are recognized by helper T cells simultaneously with antigen on the surface of B cells and MΦ.

Materials and Methods

Mice. With the following exception, all mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. B10.AM(S) (*H-2*^{s}) mice were bred in our own colony from breeding pairs kindly provided by Dr. Jack Stimpling, McLaughlin Institute, Great Falls, Mont.

Cultures. Mouse spleen cells were cultured by the methods of Mishell and Dutton (12) with the following modifications. Eagle's minimal essential medium (modified F-14) for suspension culture without glutamine or $NaHCO_3$ was purchased in powdered form from Grand Island Biological Co., Grand Island, N.Y. During preparation the medium was supplemented with nonessential amino acids (12), sodium pyruvate (12), and 2.2 g $NaHCO_3$/liter. It was then sterilized by filtration and stored at 4°C. Just before use the medium was supplemented with 30 mg glutamine/liter, 50,000 U penicillin/liter, 50 mg streptomycin/liter, 50 ml of fetal calf serum (FCS)/liter, and 2-mercaptoethanol to a final concentration of 5×10^{-5} M (13). The medium was then used within 24 h. In the present study FCS lot 90092 purchased from Microbiological Associates, Bethesda, Md., was used throughout. Cultures were incubated at 37°C in an atmosphere of 10% CO_2/90% air on a rocking platform (Bellco Glass, Inc., Vineland, N. J.). Cells were cultured in 0.5 ml in Linbro FB16-24TC culture trays (Linbro Chemical Co., New Haven, Conn.) at concentrations of 3–6 $\times 10^6$ cells/culture well. Cultures were fed daily with 0.1 vol nutrient cocktail (12).

Antigens. SRBC from a single animal were obtained from Bellwhether Farms, Palmyra, N. Y. BRBC and horse RBC (HRBC) from single animals were purchased from the Colorado Serum Co., Denver, Colo. Trinitrophenylated (TNP) RBC were prepared by the method of Rittenberg and Pratt (14) as modified by Kettman and Dutton (15). Heavily conjugated RBC were used as the immunogen in culture and lightly conjugated RBC, as the antigen in the hemolytic plaque assay.

Immunizations. Mice were primed by intravenous injection with 8–20 $\times 10^5$ SRBC in balanced salt solution (BSS) (8, 9, 16). Spleens were removed 4 days later as a source of SRBC-primed helper T cells. In vitro immunizations were 2×10^6 TNP-RBC/ml culture medium.

Preparation of T Cells. Splenic T cells were prepared using nylon fiber columns by modification of the methods of Greenwalt et al. (17), Julius et al. (18), and Greaves and Brown (19). Nylon staple fiber (3.0 denier, 1½ inch, type 200) was a generous gift from E. I. Du Pont de Nemours & Co., Wilmington, Del. Before use the fiber was washed for 1 h in a solution of 1% Liqui-Nox (Alconox Inc., New York) at 80°C (60 g of fiber/liter). After thorough washing with distilled water, the fiber was soaked in 1 N HCl for 1 h at room temperature and washed thoroughly with distilled water. The fiber was then dried and carded using hand wool carders (Eager Weavers, Rochester, N. Y). The carded fiber was placed in columns made from disposable syringes (120 mg/ml). 5 ml of bed volume was used for each 1–1.5 $\times 10^8$ spleen cells (approximately one spleen) to be prepared. After sterilization by autoclaving the columns were soaked in 5% FCS in BSS at 37°C and incubated for at least 30 min at 37°C. After washing with one column volume of 5% FCS in BSS at 37°C, spleen cells were applied to the column in two-thirds column volume of the same buffer. After an additional 30 min at 37°C the nonadherent cells were eluted from the column in two column volumes of 5% in BSS at 37°C. Routinely, 12–15% of the applied cells was recovered. These cells were 90–100% T cells as judged by lysis with anti-T-cell serum and complement and accounted for ≅ 50% of the applied T cells. They were severly depleted of functional MΦ and B cells (see Table I).

In some experiments, double-sized columns were prepared. In these cases the spleen cells were loaded in one-third the column volume, moved farther into the column after 30 min using another one-third column volume of 5% FCS in BSS, and finally after an additional 30 min, eluted with one column volume of 5% FCS in BSS. Recoveries were comparable to those above and the isolated T cells had properties indistinguishable from those of T cells passed through single-sized columns.

TABLE I

Presence of Functional MΦ in Adherent Peritoneal Cells and in Irradiated, Anti-T-Cell Serum Plus C'-Treated Normal Spleen Cells

Exp.	B cells*	T cells‡	Source of MΦ		Antigen	Anti-TNP PFC/culture (day 4)
			B10.A Peritoneal cells (adherent fraction)	Irradiated, anti-T-cell serum + C'-treated normal B10.A spleen cells		
I	4.0×10^6	1×10^6	–	–	TNP-SRBC	20 ± 5§
	–	1×10^6	1×10^5	–	TNP-SRBC	<2
	4.0×10^6	1×10^6	1×10^5	–	TNP-SRBC	220 ± 15
II	2.5×10^6	1×10^6	–	–	TNP-SRBC	40 ± 10
	–	1×10^6	–	2.5×10^6	TNP-SRBC	<2
	2.5×10^6	1×10^6	–	2.5×10^6	TNP-SRBC	270 ± 30

* Anti-T-cell serum plus C'-treated Sephadex G-10-purified B10.A spleen cells.
‡ Nylon-purified SRBC-primed CAF_1 spleen cells.
§ SEM.

Depletion of T Cells. Spleen cells were depleted of T cells as previously described using anti-T serum and complement (20). These preparations were used as a source of B cells and MΦ.

MΦ-Depleted B Cells. Spleen cells were depleted of MΦ and T cells by passage over Sephadex G-10 columns by the method of Ly and Mishell (21) followed by a treatment with anti-T serum and complement as above. Such preparations contained functional B cells.

Sources of MΦ. Two sources of MΦ were used. Adherent peritoneal cells were used in most cases. The peritoneal cavities of normal mice were washed with cold 5% FCS in BSS to remove peritoneal cells. Individual cultures received 10^5 cells in 0.5 ml of medium. After 24 h, nonadherent cells were removed by aspiration and spleen cells cultured on the remaining macrophages which were uniformly distributed in the culture well. In one experiment splenic MΦ were used. They were obtained from spleen cell suspensions which were depleted of T-cell activity by treatment with anti-T-cell serum and complement and of B-cell activity by irradiation (1,000 rads from a ^{60}Co source).

Both the spleen and the peritoneal cavity have been traditional sources of functional MΦ for in vitro antibody responses (21–24). In the case of peritoneal MΦ, we have previously demonstrated their ability (in our hands) to restore in vitro concanavalin A responsiveness to adherent cell-depleted splenic T cells (25) and have shown in several studies (10, 11) that these cells, when pulsed with antigen, can be used to prime in vivo antigen-specific, *H-2*-restricted helper T cells.

As an additional check on the immunological capabilities of our MΦ preparations, perhaps more relevant to the present study, we tested their ability (in our hands) to restore the in vitro response of MΦ-depleted T and B cells to the antigen, TNP-SRBC. The results of these experiments are shown in Table I. Adherent cell-depleted T and B cells made little or no response to TNP-SRBC. The addition of either the adherent fraction of peritoneal cells or irradiated, anti-T-cell serum plus complement-treated normal spleen cells as a source of MΦ restored the response. It should be noted that in these and in all experiments reported here the culture medium was supplemented with 2-mercaptoethanol, so that, whatever the function of MΦ in this response, it is not a function which can be performed by 2-mercaptoethanol.

Plaque-Forming Cell (PFC) Assay. After 4 days of culture, three identical culture wells were pooled and assayed in duplicate for PFC using the slide modification (12) of the Jerne hemolytic plaque assay (26). Parallel determinations were made using TNP-HRBC and HRBC, and the difference recorded as the number of anti-TNP PFC.

Assay of Helper T-Cell Activity. Helper T-cell activity was titrated as previously described (9). Briefly, cultures were prepared which contained a constant number of B cells and MΦ and

varying numbers of SRBC-primed helper T cells. The cultures were immunized with either TNP-SRBC or TNP-BRBC, and after 4 days, the number of anti-TNP PFC/culture determined. 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 this line was taken as the activity of the helper population. To compare the cross-reactivity of SRBC-primed helper cells with BRBC, a comparison of slopes obtained with the two TNP-RBC was made (see Fig. 1).

Since in all these determinations we were interested only in the helper activity present as a result of SRBC priming, some correction had to be made to take into account the "background" helper activity specific for most RBC antigens present in unimmunized animals. This was accomplished in two ways.

When measurements were to be made with totally syngeneic cells, cultures were constructed to contain various portions of normal and primed unseparated spleen cells keeping the total cell number per culture constant. In this way all cultures contained constant number of TNP-specific B cells and background helper activity but varying numbers of primed helper cells. Thus, the titration slopes reflected only the activity of the primed helper cells (9).

When measurements were made using F_1 T cells with B cells and MΦ carrying parental *H-2* haplotypes, it was necessary to remove T cells from the B cell/MΦ preparation to avoid an allogeneic interaction with the F_1 T cells. Likewise, B cells and MΦ had to be removed from the F_1 T cells to prevent their contribution to the response. In this case varying numbers of F_1 T cells were added to a constant number of B cells and MΦ. Thus, the background helper activity contaminating the primed F_1 T cells was also a variable and contributed to the total activity seen. To correct for this activity, parallel titrations were performed with unprimed F_1 T cells and the difference between this activity and that seen with primed F_1 T cells calculated as the helper activity due to priming (Figs. 3, 4, 6).

Results

Genetic Control of the Response of Helper T Cells to RBC Antigens. In a large number of experiments various mouse strains, including *H-2* congenic strains of the B10 or C3H background, were immunized with SRBC. After 4 days, the SRBC-primed spleen cells were titrated for primed helper activity as described in the Materials and Methods using both TNP-SRBC and TNP-BRBC as antigens. Three representative pairs of titrations are shown in Fig. 1. Titrations were initially linear so that their slopes were indications of the relative activities of the primed helper cells. In each case helper activity was detected using TNP-SRBC as the antigen; however, these SRBC-primed helper cells did not cross-react equally well in all cases with TNP-BRBC. A cross-reaction with TNP-BRBC of ≅ 50% was seen in DBA/2 mice; the cross-reaction was only 28% in C3H.SW mice and virtually absent in B10.A(2R) mice.

Fig. 2 summarizes the results of 40 pairs of titrations similar to those in Fig. 1, utilizing 12 mouse strains. The average cross-reactivity of SRBC-primed helper cells with TNP-BRBC observed in each strain is shown. The strains fell roughly into three groups: (*a*) those showing little (0–15%) cross-reactivity: B10.A, B10.A(2R), C3H/He, and CBA; (*b*) those showing intermediate (15–30%) cross-reactivity: C57BL/6, C57BL/10(B10), C3H.SW, and DBA; and (*c*) those showing high (>30%) cross-reactivity: DBA/2, BALB/c, B10.D2, and B10.AM(S).

The data demonstrate that the degree of cross-reaction is controlled by a gene(s) linked to the *H-2* complex since: (*a*) strains of the same *H-2* type show approximately the same level of cross-reaction; and (*b*) strains congenic at *H-2* of the B10 and C3H background show different levels of cross-reaction depending on *H-2* haplotype. Therefore, we concluded that in the mouse the ability of

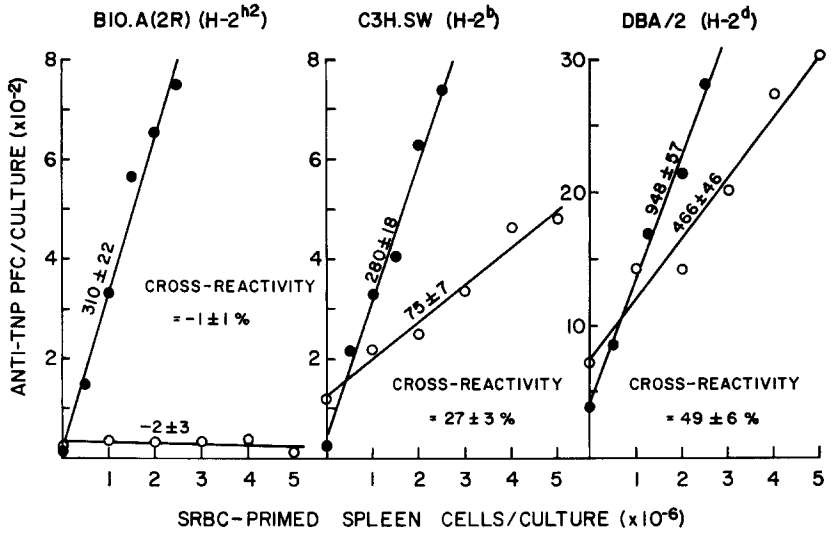

FIG. 1. Titration of SRBC-primed helper activity using TNP-SRBC and TNP-BRBC. Groups of two B10.A(2R), C3H.SW, and DBA/2 mice were primed with SRBC. After 4 days, a pooled spleen cell suspension was prepared from each group and titrated in vitro for helper activity as described in the Materials and Methods using either TNP-SRBC (●) or TNP-BRBC (○) as the antigen and a cell concentration of 5×10^6/culture. The number of anti-TNP PFC/culture was determined on day 4 and plotted vs. the number of SRBC-primed cells/culture. The least squares method was used to determine the initial slope ± SE of the titration line. The percent cross-reactivity of SRBC-primed helper cells with BRBC was calculated as the slope obtained with TNP-BRBC as antigen divided by the slope obtained with TNP-SRBC as antigen × 100.

helper T cells to respond to a determinant(s) shared between SRBC and BRBC was controlled by an *H-2*-linked immune response (*Ir*) gene(s). $H\text{-}2^a$, $H\text{-}2^{h2}$, and $H\text{-}2^k$ determined low responsiveness; $H\text{-}2^b$ and $H\text{-}2^q$ determined intermediate responsiveness; and $H\text{-}2^d$ and $H\text{-}2^s$ determined high responsiveness.

Since the study included two strains which had recombinant *H-2* haplotypes, it was possible to tentatively map the Ir gene(s) within the *H-2* complex. Table II lists the *H-2*-subregion types of the congenic strains of the B10 background used in these experiments. The high response seen in B10.D2 mice and the low response in B10.A mice would tentatively map the gene(s) controlling high responsiveness in $H\text{-}2^d$ mice to the *K* through *I-E* end of the *H-2* complex. Likewise, the intermediate response in B10 mice and the low response in B10.A(2R) mice would tentatively eliminate *D* as the site of the gene(s) controlling intermediate responsiveness in $H\text{-}2^b$ mice.

Mapping on the basis of such limited distribution data is, however, risky at best, especially in light of the recent discoveries that in some cases *Ir* phenotypes may be the result of interacting *Ir* genes which map in different subregions of the *H-2* complex (27, 28).

One point which became clear in these studies was that in order to observe this *H-2* control, especially when noncongenic mice were used, it was necessary to compare the activity of SRBC-primed helper cells with TNP-SRBC and with TNP-BRBC rather than simply to determine their activity with TNP-BRBC alone. This was because the absolute level of helper activity measured after

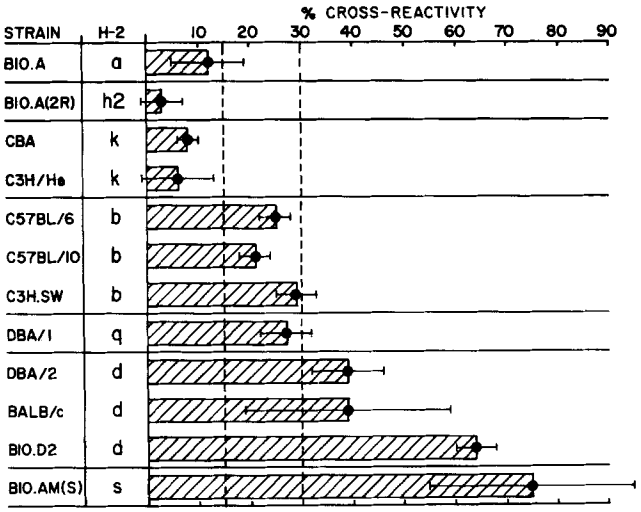

Fig. 2. An *H-2*-linked *Ir* gene(s) controlling cross-reaction of SRBC-primed helper cells with BRBC. 40 pairs of titrations were performed as in Fig. 1 using 12 different strains of mice. Cross-reactivity of SRBC-primed helper cells with BRBC was calculated as in Fig. 1. Shown are the average cross-reactivities ± SEM of three to four separate determinations with each strain. Dotted lines arbitrarily divide the strains into low, intermediate, and high responders to the shared determinant(s) on SRBC and BRBC.

SRBC priming varied considerably among the strains. This variation was not a function of *H-2*-linked genes as seen in Table III. When the absolute helper activities measured in three $H\text{-}2^d$ strains (DBA/2, BALB/c, and B10.D2) were compared, the highest activity was consistently seen in DBA/2 and the lowest in BALB/c with B10.D2 as the intermediate. Nevertheless, a comparison of the activities measured with TNP-SRBC and TNP-BRBC in the three strains allowed one to classify all three as high responders to the shared RBC determinant(s).

Of the strains listed in Fig. 2, highest absolute responses were seen in DBA/1 and DBA/2. Intermediate responses were generally seen in B10 and C3H congenics and in CBA, and BALB/c consistently gave the lowest activity.

Expression of the Ir Gene(s) in B cells. Having demonstrated *Ir* gene(s) controlling the response of helper T cells to the shared determinant(s) on SRBC and BRBC, we wished to determine in which cell type(s) the gene(s) was expressed: T cells, B cells, or MΦ. The simplest approach to this question would have been to mix T cells, B cells, and MΦ from high, intermediate, and low responder mice in various combinations in vitro. This was impossible since such experiments would mean the unavoidable mixing of T cells with allogeneic B cells and MΦ. The resulting allogeneic interactions (both positive and negative) would certainly have obscured the results.

As an alternate approach we prepared T cells from F_1 animals of parents with different *Ir* types. The two F_1 mice which were used were C57BL/6 × DBA/2 (BDF_1) (intermediate, $H\text{-}2^b$, × high, $H\text{-}2^d$, responder) and BALB/c × A/J (CAF_1) (high, $H\text{-}2^d$, × low, $H\text{-}2^a$, responder). Normal and SRBC-primed F_1 T cells were tested for helper activity using TNP-SRBC and TNP-BRBC. T cell-depleted spleen cells from *H-2*-congenic mice of the B10 background

TABLE II
Mapping of Ir-Genes(s)

Strain	H-2 Subregions									Cross-reactivity
	K	*I-A*	*I-B*	*I-J*	*I-E*	*I-C*	*S*	*G*	*D*	
										%
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*d*	64 ± 4*
B10.A	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*	*d*	12 ± 7‡
B10.A(2R)	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*?*	*b*	3 ± 4‡
B10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	21 ± 3‡
B10.AM(S)	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	*s*	75 ± 20‡

* Average ± SEM of four separate determinations.
‡ Average ± SEM of three separate determinations.

TABLE III
Non-H-2 Influence on SRBC-Specific Helper Activity

Strain	SRBC-specific helper activity (PFC/culture/10^6 primed cells)*	BRBC Cross-reacting helper activity (PFC/culture/10^6 primed cells)*	Cross-reactivity*
			%
DBA/2	956 ± 200	350 ± 49	39 ± 7
B10.D2	240 ± 43	151 ± 19	64 ± 4
BALB/c	135 ± 21	52 ± 20	39 ± 20

* Average ± SEM of four separate determinations.

carrying one or the other parental *H-2* haplotypes were used as a source of B cells and MΦ in the helper cell assay.

Thus, allogeneic interactions were avoided since the F_1 T cells were incapable of recognizing either the *H-2* antigens or in this case the *Mls* antigens of the B cells and MΦ. (C57BL/6, C57BL/10, BALB/c, B10.A, and B10.D2 are identical at *Mls*.)

We predicted that if the *Ir* gene(s) was expressed only in T cells, then the F_1 T cells would cross-react equally well with BRBC using B cells and MΦ of either parental *H-2* haplotype. However, if the gene(s) was expressed in B cells and/or MΦ, then the extent of cross-reaction would be a reflection of the *H-2* haplotype of the B cells and MΦ used.

The results of two experiments of this type are summarized in Figs. 3 and 4. In Fig. 3, when BDF_1 T cells were tested with B10 B cells and MΦ, intermediate cross-reaction of SRBC-primed T cells with BRBC was seen. However, high cross-reaction was seen when B10.D2 B cells and MΦ were used. In Fig. 4, CAF_1 T cells showed high cross-reaction when tested with B10.D2 B cells and MΦ, but low cross-reaction with B10.A B cells and MΦ. Each of these experiments was repeated twice with similar results. The results for the six experiments are summarized in Fig. 5, where the average cross-reactivity for each cell combination is shown.

We concluded from these experiments that for a given SRBC-primed F_1 cell preparation, the extent of cross-reaction observed with BRBC was predicted by the *Ir* type of the B cell and/or MΦ present in the helper cell assay.

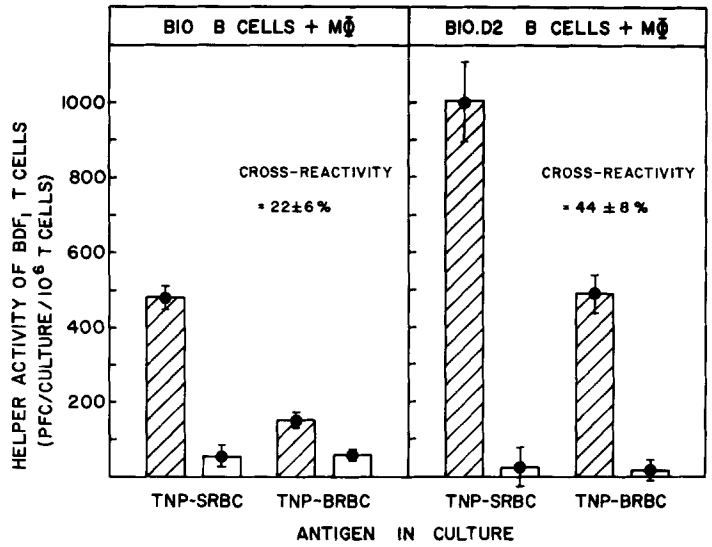

FIG. 3. Expression of the *Ir* gene(s) in B cells and/or MΦ: BDF_1 cells tested on B10 and B10.D2 cells and MΦ. T cells were isolated from the spleens of groups of three SRBC-primed (hatched bars) or normal (open bars) BDF_1 (*H-2*^{b} × *H-2*^{d}) mice. These T cells were titrated (0–2 × 10^6/culture) in vitro for helper activity using B cells and MΦ (4 × 10^6/culture) prepared from either B10 (*H-2*^{b}) or B10.D2 (*H-2*^{d}) spleen cells. Both TNP-SRBC and TNP-BRBC were used as antigens. For each titration the number of anti-TNP PFC/culture was plotted vs. the number of T cells added and the initial linear slope determined by the least squares method. This slope ± SE is shown for each titration. The percent cross-reaction of SRBC-primed helper cells with BRBC was calculated as: (slope obtained with primed T cells minus the slope obtained with normal T cells when TNP-BRBC was the antigen) divided by (slope obtained with primed T cells minus the slope obtained with normal T cells when TNP-SRBC was the antigen) × 100.

In an attempt to determine whether the *Ir*-gene control was expressed in the in vitro helper T-cell assay in the B cells, the MΦ, or both, we performed experiments, such as those illustrated in Fig. 6. CAF_1 T cells were titrated for helper activity using B10.A (low responder) B cells and MΦ and either TNP-SRBC or TNP-BRBC as antigen. The assay cultures contained, in addition, the adherent cells (MΦ) from 10^5 peritoneal cells from either B10.A (low responder) or B10.D2 (high responder) mice. If the *Ir* gene(s) was expressed in MΦ but not B cells, then the low cross-reactivity seen with B10.A B cells and MΦ should change to high cross-reactivity with the addition of B10.D2 MΦ. The results showed that regardless of the type of additional MΦ present in the assay cultures, low cross-reactivity was seen between SRBC-primed T cells and BRBC. This experiment was repeated twice. The average cross-reactivity observed in the three experiments with additional B10.A MΦ was 10 ± 4% and with B10.D2 MΦ 13 ± 2%.

In a fourth experiment rather than using peritoneal cells as the source of MΦ, we used B10.A or B10.D2 splenic MΦ prepared as described in the Material and Methods by irradiation of T cell-depleted splenic B cells and MΦ. Cultures contained 2.5 × 10^6 B10.A B cells and MΦ plus 2.5 × 10^6 of either irradiated preparation. The cross-reactivity was 9 ± 2% with irradiated B10.A cells and 12 ± 3% with B10.D2 cells.

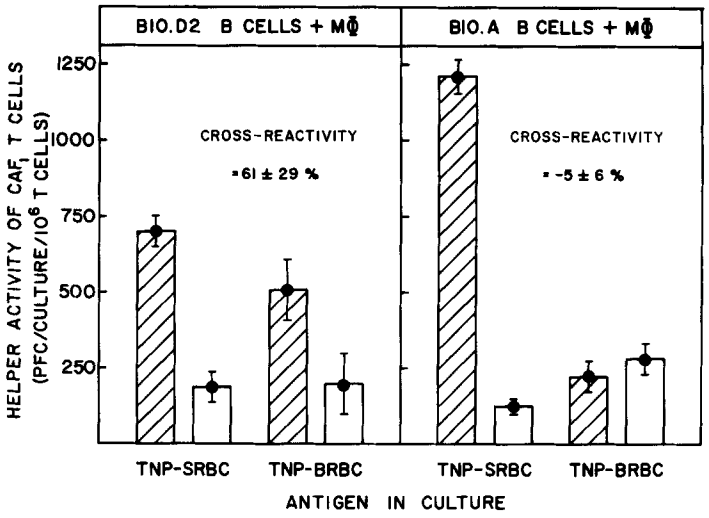

FIG. 4. Expression of the *Ir* gene(s) in B cells and/or MΦ: CAF_1 T cells tested on B10.D2 and B10.A B cells and MΦ. Same as Fig. 3 except that CAF_1 ($H\text{-}2^d \times H\text{-}2^a$) T cells and either B10.D2 ($H\text{-}2^d$) or B10.A ($H\text{-}2^a$) B cells and MΦ were used.

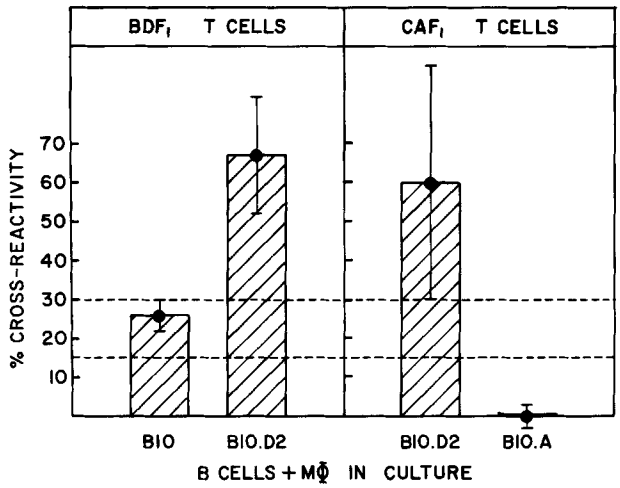

FIG. 5. Expression of *Ir* gene(s) in B cells and/or MΦ: Summary. The experiments described in Figs. 3 and 4 were each performed a total of three times. The average cross-reactivities ± SEM are shown for each cell combination.

We concluded from these experiments that the *Ir*-gene control of this response was not simply at the level of T-cell/MΦ interaction, since if this were so, the addition of B10.D2 MΦ to the CAF_1 T cell/B10.A B cell and MΦ cultures should have increased the cross-reactivity observed. Although these experiments do not eliminate the possibility that the *Ir* gene(s) is expressed in the MΦ (or the T cell), they do indicate that at least some other cell type must also obligatorily express the *Ir* gene(s) in order for cross-reactivity to be observed. It would appear that the responding B cell is the only likely candidate for this cell type.

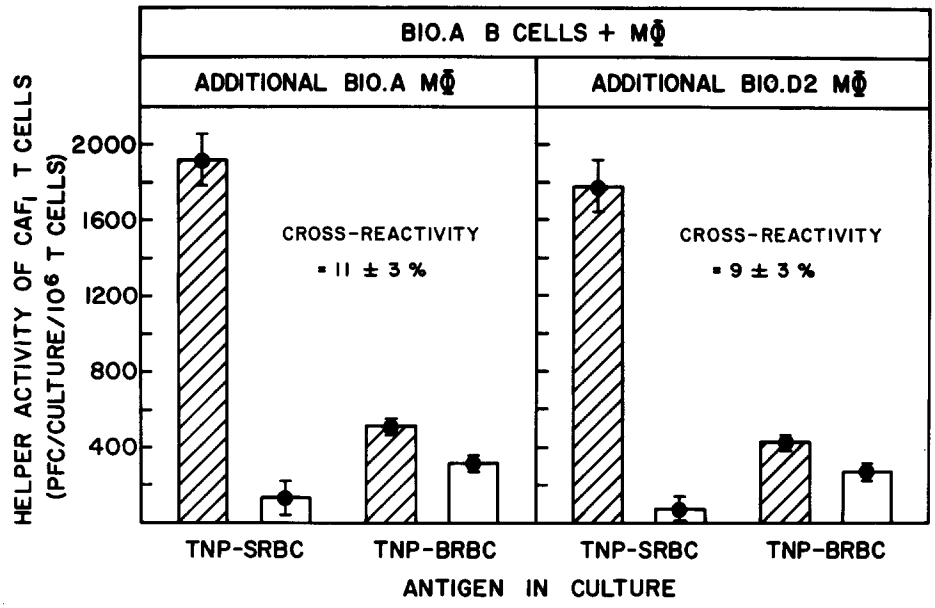

FIG. 6. Expression of the Ir gene(s) in B cells at least. SRBC-primed (hatched bars) and normal (open bars) CAF_1 cells were titrated for helper activity as in Fig. 3 using TNP-SRBC and TNP-BRBC as antigens and B10.A B cells and MΦ. However, cultures contained in addition the adherent portion of 10^5 peritoneal cells from either B10.A or B10.D2 mice. Slopes of the titration lines are shown. The cross-reactivities were calculated as in Fig. 3.

Discussion

In many species it appears to be a general phenomenon that genes (*Ir* genes) within the MHC can control the immune response to specific antigens (12). Although much controversy concerning the mode of action of *Ir* genes still exists, there seems to be a consensus on at least two points. Firstly, there is apparent antigen specificity in the effects of these genes. Secondly, the ultimate effect of an *Ir* gene is on the expression of the activity of T cells.

Originally, the simplest hypothesis to explain these observations was the involvement of MHC gene products, particularly those of the *I* region, in the structure of the T-cell receptor for antigen, i.e., the expression of *Ir* genes in T cells. This view was given further support by the difficulty of demonstrating immunoglobulin on T cells (29), the inhibition of T-cell proliferation in vitro with anti-Ia sera (30), and the demonstration of an Ia-positive, T cell-derived, antigen-specific factor (31).

However, over the years evidence steadily appeared, which argued against this simple hypothesis. The first was the surprising finding that products of the *I* region were detected easily on B cells but only with great difficulty on T cells (32, 33). At about the same time, Katz and co-workers (3–5) and Rosenthal and Shevach (6, 7) demonstrated that the ability of in vivo-primed T cells to collaborate with either B cells or MΦ, respectively, apparently required MHC identity between the collaborating cells. These studies included experiments which showed that for antigens the response to which was under *Ir*-gene control, antigen-primed T cells from F_1 animals of high × low responder

parents collaborated only with B cells or MΦ from the high responder, not the low responder parent. These experiments clearly demonstrated the expression of *Ir* genes in cells other than T cells and were originally interpreted to mean that in order for effective collaboration to take place the cells involved must share and express certain MHC genes.

However, even this conclusion had to be modified in light of experiments which appeared concerning the recognition of antigenic targets by cytotoxic T cells. The conclusion reached in a large number of laboratories (34–37) was that cytotoxic T cells recognized target antigens in combination with products of the MHC. In the mouse these products were primarily of the *K* and/or *D* regions. Thus, when T cells were immunized with antigen associated with a particular MHC type, the effectors generated were capable of killing effectively only antigen-bearing targets of the same MHC type. These results quickly led to a reinterpretation of the T-cell/B-cell and T-cell/MΦ experiments. Since in all these experiments T cells had been primed in a syngeneic environment, it was possible that they had been selected for their ability to recognize the antigen in association with their own MHC gene products. This selection might account for their inability to collaborate with cells of other MHC types. For *Ir* gene-controlled responses the control might be at the level of the formation or recognition of an *Ir*-gene product/antigen complex. Indeed, recently several reports have appeared in which T cells primed in a semiallogeneic or allogeneic environment were shown capable of collaborating with allogeneic B cells or MΦ (38–41).

Although attention has focussed in turn on each of these interpretations of the mode of action of MHC-linked *Ir* genes, at present, none can be eliminated from consideration. Reports continue to appear of antigen-specific T-cell products bearing MHC alloantigens (42–45). In addition, there are numerous recent reports in which it appears that in order for effective interactions to take place between T and B cells, MΦ, or other T cells, the interacting cells must be genetically related (43, 44, 46–50). And yet simultaneously, experiments are being reported which are most easily explained by the conclusion that T cells are restricted to recognizing cell-bound antigen in association with products of the MHC (51, 52, and reviewed in 53). It may be that given the size and complexity of the MHC there is room for contributions from all three mechanisms to the immune response.

As stated above, the approach that we and others have taken in an attempt to distinguish among these mechanisms has been to determine which cell types need express the relevant MHC genes in order for the MHC control to be observed. In previous studies (10, 11) we have shown that F_1 mice whose parents differed at *H-2* could be primed with keyhole limpet hemacyanin (KLH) bound to MΦ-bearing parental *H-2* haplotypes. The resulting primed F_1 helper T cells were shown to cooperate preferentially in vitro with B cells and MΦ bearing the same parental *H-2* haplotype as the original priming MΦ. In the present study we demonstrated that the response of mice to determinants shared between SRBC and BRBC was under the control of an *H-2*-linked *Ir* gene(s). When helper T cells from F_1 animals whose parents had different *Ir* types were tested in vitro with B cells and MΦ of parental *Ir* types, the extent of response to the SRBC/BRBC-cross-reacting determinants was predicted by

the *Ir* type of the responding B cells and MΦ (Fig. 5).

These results established that *H-2*-linked genes can control helper T-cell activity although they are expressed in non-T cells, and would apparently eliminate the possibility that the only site of action of these genes is in the helper T-cell antigen receptor. In this respect our results are in agreement with those of several other laboratories (3–7, 41, 46, 49–52). However, a more controversial point has been the question of which non-T cells express the genes. Can B cells express the genes during T cell/B cell interactions or can all of the observed phenomena be explained on the basis of the expression of the genes in MΦ during T cell/MΦ interactions?

The work of Shevach and co-workers (6, 7, 51, 52), Erb and Feldmann (46), Pierce et al. (41), and ourselves (10, 11), has established that T-cell interaction with antigen bound to the MΦ surface is under the control of MHC-linked genes expressed in the MΦ. In at least one of these studies (41) all of the control was shown to be at the level of helper T-cell/MΦ interactions, with the MHC type of the responding B cell irrelevant. In addition, Bechtol et al. (38) have reported that in the response to (T,G)AL in tetraparental mice produced from responder and nonresponder parents, B cells of nonresponder origin respond well. Experiments of this type have led some to conclude that MHC control of helper T-cell activity is not evident at the level of T-cell/B-cell interactions, but only at the level of T-cell/MΦ interactions.

On the other side of the argument the strongest evidence for the expression in B cells of MHC-linked genes controlling helper T-cell function has come from the work of Katz and co-workers (3–5, 49, 50) and Taussig, Munro, and co-workers (31, 54). In the first study, T and B cells from mice which differed at the *I* region of *H-2* were shown to fail to cooperate in irradiated F_1 recipients. In the second study, *Ir* genes determining the response to (T,G)AL were shown to be expressed in B cells and control the ability of the B cells to respond in irradiated recipients to an antigen-specific helper factor. However, both of these studies were performed in vivo where the contribution of MΦ to the response was difficult to quantitate. The possibility has been raised that T cell/MΦ interactions rather than T-cell/B-cell interactions may have in some undetected way contributed to the results observed.

Our experiments have been performed in vitro where one might expect to have more control over the types of cells participating in the response. In neither this (Fig. 6) nor our previous (10, 11) studies have we been able to circumvent MHC control of T-cell/B-cell interaction by the addition of MΦ of the appropriate *H-2* (*Ir*) type to the cultures. In the present work both splenic and peritoneal MΦ were used and control experiments were performed to assure that these MΦ were functional in this in vitro response. Therefore, we interpret our results to support the view that MHC-linked genes expressed in B cells can control T-cell/B-cell interaction.

Our experiments do not, however, exclude the possibility that MHC-linked genes expressed in MΦ are important not only in the initial priming of helper T cells in vivo, but also in the subsequent interaction of the primed helper cells with antigen in vitro. The responses to the antigens which we have studied thus far, (TNP-KLH, TNP-RBC) are certainly MΦ dependent in vitro even in the presence of 2-mercaptoethanol (e.g., Table I). It is possible and perhaps

even probable that MΦ-bound antigen serves to restimulate the primed helper T cells before their interaction with B cells. If so, it seems likely that *H-2* genes expressed in the MΦ would control this restimulation. We are currently testing this possibility.

Our experiments also do not exclude the direct expression of *H-2* genes in helper T cells, as has been suggested by the work of Katz and co-workers (3–5, 49, 50). Since in all of the experiments we have reported thus far, F_1 mice have been used as the source of helper T cells, we have not directly addressed the question of the expression of these genes in T cells. However, if such were the case, our results indicating separate subpopulations of F_1 T cells interacting with B cells and MΦ of parental *H-2* haplotypes, would suggest that a phenomenon similar to allelic exclusion must be occurring in the F_1 T cells resulting in selective expression of *H-2* genes in different subpopulations of T cells.

In summary, we feel the simplest interpretation of our results is as follows. Helper T cells respond only to cell-bound antigen. Their initial interaction with antigen takes place on the surface of MΦ (and perhaps other cell types) where they are clonally selected on the basis of their ability to recognize both the antigen and products of the MHC expressed on the MΦ surface. This recognition may be through a single or separate receptors on the T cells and may involve MHC genes expressed in the T cell. For large multideterminant antigens such as KLH presumably many MHC gene products can be involved. For structurally restricted antigens the response to which is under *Ir*-gene control, a single or small number of MHC genes are involved. After selection and clonal expansion, primed helper T cells function only when they recognize simultaneously both the antigen and the MHC gene products involved in their original selection. This recognition may take place on a MΦ, but for an effective B-cell response, the simultaneous recognition must at least take place on the B-cell surface.

As stated above this interpretation is not consistent with all of the data reported in the literature. Moreover, modifications may be required as new information comes to light. It does, however, represent our current working hypothesis and will be useful in the design of future experiments.

Summary

The ability of murine helper T cells primed to the antigen, sheep erythrocytes (SRBC) to cross-react with burro erythrocytes (BRBC) in the in vitro anti-trinitrophenol (TNP) response to TNP-RBC was shown to be under genetic control. Although non-*H-2* genes were shown to influence the absolute level of helper activity assayed after SRBC priming, the extent of cross-reaction of SRBC-primed helpers with BRBC was shown to be controlled by an *H-2*-linked *Ir* gene(s). *H-2* haplotypes were identified which determined high, intermediate, or low response to the cross-reacting determinants and the gene(s) controlling the cross-reaction tentatively mapped to the *K* through *I-E* end of the *H-2* complex.

Helpers primed in F_1 mice of high × intermediate or high × low responder parents were tested for cross-reaction using B cells and macrophages (MΦ) of parental haplotypes. In each case the extent of cross-reaction was predicted by

the *H-2* haplotype of the B cells and MΦ, establishing the expression of the *Ir* gene(s) in B cells and/or MΦ at least, but not ruling out its expression in T cells as well. The low cross-reaction seen when T cells from F_1 mice of high × low responder parents were tested on low responder B cells and MΦ was not increased by the presence of high responder MΦ, indicating the *Ir* gene(s) is expressed in the B cell at least although it may be expressed in MΦ as well. These and our previously reported experiments are consistent with the hypothesis that helper T cells recognize antigen bound to the surface of B cells and MΦ in association with the product(s) of *Ir* gene(s) expressed on the B cell and MΦ.

We thank Lee Harwell and George Berry for their excellent technical assistance. We thank the Animal Tumor Research Facility and the Biostatistics Facility of the University of Rochester Cancer Center for their help in supplying tumor cells for antiserum and complement adsorption and in developing computer programs for the statistical handling of our data respectively. We are grateful to Dr. Christopher Lawrence (Department of Radiation Biology and Biophysics, University of Rochester) for the use of his ^{60}Co irradiator.

Received for publication 6 June 1977.

References

1. Benacerraf, B., and H. O. McDevitt. 1972. The histocompatibility-linked immune response genes. *Science (Wash. D. C.)*. 175:273.
2. Benacerraf, B., and D. H. Katz. 1975. The histocompatibility-linked immune response genes. *Adv. Cancer Res.* 21:121.
3. Katz, D. H., T. Hamaoka, M. E. Dorf, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. III. Demonstration that the H-2 gene complex determines successful physiologic lymphocyte interactions. *Proc. Natl. Acad. Sci. U.S.A.* 70:2624.
4. Katz, D. H., T. Hamaoka, M. E. Dorf, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* 137:1405.
5. 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.
6. Rosenthal, A. S., and E. M. Shevach. 1973. The function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* 138:1194.
7. Shevach, E. M., and A. S. Rosenthal. 1973. The function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of the immune response. *J. Exp. Med.* 138:1213.
8. Hoffmann, M., and J. W. Kappler. 1972. The antigen specificity of thymus-derived helper cells. *J. Immunol.* 108:261.
9. Hoffmann, M., and J. W. Kappler. 1973. Regulation of the immune response. II. Qualitative and quantitative differences between thymus- and bone marrow-derived lymphocytes in the recognition of antigen. *J. Exp. Med.* 137:721.
10. Kappler, J. W., and P. C. Marrack. 1976. Helper T cells recognize antigen and macrophage surface components simultaneously. *Nature (Lond.)*. 262:797.
11. Kappler, J. W., and P. C. Marrack. 1977. Simultaneous recognition of carrier antigens and products of the *H-2* complex by helper T cells. *In* Regulation of the

Immune System. E. Sercarz and L. Herzenberg, editors. Academic Press, Inc., New York. In press.
12. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
13. Click, R. E., L. Benck, and B. J. Alter. 1972. Enhancement of antibody synthesis *in vitro* by mercaptoethanol. *Cell Immunol.* **3**:156.
14. 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.
15. Kettman, J. R., and R. W. Dutton. 1970. An *in vitro* primary immune response to 2, 4, 6-trinitrophenyl substituted erythrocytes: Response against carrier and hapten. *J. Immunol.* **104**:1558.
16. Falkoff, R., and J. Kettman. 1972. Differential stimulation of precursor cells and carrier-specific thymus-derived cell activity in the *in vivo* response to heterologous erythrocytes in mice. *J. Immunol.* **108**:54.
17. Greenwalt, T. J., M. Gajewski, and J. L. McKenna. 1962. A new method for preparing buffy coat-poor blood. Transfusion (*Phila.*). **2**:221.
18. 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.
19. Greaves, M. F., and G. J. Brown. 1974. Purification of human T and B lymphocytes. *J. Immunol.* **112**:420.
20. 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.
21. 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.
22. Hoffmann, M. 1970. Peritoneal macrophages in the immune response to SRBC *in vitro*. *Immunology.* **18**:789.
23. Hartmann, K., R. W. Dutton, M. McCarthy, and R. Mishell. 1971. Cell components in the immune response. II. Cell attachment separation of immune cells. *Cell. Immunol.* **1**:182.
24. Mosier, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science* (*Wash. D. C.*). **158**:1573.
25. Harwell, L., J. W. Kappler, and P. Marrack. 1976. Antigen-specific and non-specific mediators of T cell/B cell cooperation. III. Characterization of the non-specific mediator(s) from different sources. *J. Immunol.* **116**:1379.
26. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody producing cells. *Science* (*Wash. D. C.*). **140**:405.
27. Dorf, M. E., and B. Benacerraf. 1975. Complementation of *H-2* linked *Ir* genes in the mouse. *Proc. Natl. Acad. Sci. U.S.A.* **72**:3671.
28. Melchers, I., and K. Rajewsky. 1976. *H-2* linked immune response genes: multiplicity and functional complementation. *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. 283.
29. Vitetta, E., C. Bianco, V. Nussensweig, and J. Uhr. 1972. Cell surface immunoglobulin. IV. Distribution among thymocytes, bone marrow cells, and their derived populations. *J. Exp. Med.* **136**:81.
30. Shevach, E. M., W. E. Paul, and I. Green. 1972. Histocompatibility-linked immune response gene function in guinea pigs. Specific inhibition of antigen-induced lymphocyte proliferation by alloantisera. *J. Exp. Med.* **136**:1207.
31. 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.

32. Sachs, D., and J. L. Cone. 1973. A mouse B cell alloantigen determined by gene(s) linked to the major histocompatibility complex. *J. Exp. Med.* 138:1289.
33. 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.
34. Gordon, R. D., E. Simpson, and L. E. Samuelson. 1975. *In vitro* cell-mediated immune response to male specific (H-Y) antigen in mice. *J. Exp. Med.* 142:1108.
35. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* 142:1349.
36. Zinkernagel, R. M., and P. C. Doherty. 1975. *H-2* compatibility requirement for T cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures coded from *H-2* or *H-2D*. *J. Exp. Med.* 141:1427.
37. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the *H-2K* and *H-2D* serological regions of the major histocompatibility complex. *J. Exp. Med.* 141:1348.
38. Bechtol, K. B., J. H. Freed, L. A. Herzenberg, and H. O. McDevitt. 1974. Genetic control of the antibody response to poly-L-(Try, Glu)-poly-D,L-Ala--poly-L-Lys in C3H ↔ CWB tetraparental mice. *J. Exp. Med.* 140:1660.
39. Waldmann, H., H. Pope, and A. J. Munro. 1975. Cooperation across the histocompatibility barrier. *Nature (Lond.).* 258:728.
40. Von Boehmer, H., L. Hudson, and J. Sprent. 1975. Collaboration of histoincompatible T and B lymphocytes using cells from tetraparental bone marrow chimeras. *J. Exp. Med.* 142:989.
41. 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.
42. Tada, T., M. Taniguchi, and C. S. David. 1976. Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. IV. Special subregion assignment of the gene(s) that codes for the suppressive T-cell factor in the *H-2* histocompatibility complex. *J. Exp. Med.* 144:713.
43. Taniguchi, M., T. Tada, and T. Tokuhisa. 1976. Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. III. Dual gene control of the T-cell mediated suppression of the antibody response. *J. Exp. Med.* 144:20.
44. Theze, J., J. A. Kapp, and B. Benacerraf. 1977. Immunosuppressive factor(s) extracted from lymphoid cells of nonresponder mice primed with L-glutamic acid^{60}-L-alanine ^{30}-L-tyrosine ^{10}(GAT). III. Immunochemical properties of the GAT-specific suppressive factor. *J. Exp. Med.* 145:839.
45. McDougal, J. S., and D. S. Gordon. 1977. Generation of T-helper cells *in vitro*. I. Cellular and antigen requirements. *J. Exp. Med.* 145:839.
46. 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.
47. Rich, S. S., and R. R. Rich. 1975. Regulatory mechanisms in cell-mediated immune responses. II. A genetically restricted suppressor of mixed lymphocyte reactions released by alloantigen activated spleen cells. *J. Exp. Med.* 142:1391.
48. McDevitt, H. O., T. L. Delovitch, J. L. Press, and D. B. Murphy. 1976. Genetic and functional analysis of the Ia antigens: their possible role in regulating the immune response. *Transplant. Rev.* 30:197.
49. Katz, D. H., N. Chiorazzi, J. McDonald, and L. R. Katz. 1976. Cell interactions

between histocompatible T and B lymphocytes. IX. The failure of histoincompatible cells is not due to suppression and cannot be circumvented by carrier-priming T cells with allogeneic macrophages. *J. Immunol.* 117:1853.

50. Katz, D. H., M. E. Dorf, and B. Benacerraf. 1976. Control of T-lymphocyte and B-lymphocyte activation by two complementing Ir-GLΦ immune response genes. *J. Exp. Med.* 143:907.
51. Paul, W. E., E. M. Shevach, S. Pickeral, D. W. Thomas, and A. S. Rosenthal. 1977. Independent populations of primed F_1 guinea pig T lymphocytes respond to antigen-pulsed parental peritoneal exudate cells. *J. Exp. Med.* 145:618.
52. Thomas, D. W., and E. M. Shevach. 1977. Nature of the antigen complex recognized by T lymphocytes. II. T-cell activation by direct modification of macrophage histocompatibility antigens. *J. Exp. Med.* 145:907.
53. Paul, W. E., and B. Benacerraf. 1977. Functional specificity of thymus-dependent lymhocytes. *Science* (*Wash. D. C.*). 195:1293.
54. Munro, A., and M. Taussig. 1975. Two genes in the major histocompatibility complex control immune response. *Nature* (*Lond.*). 256:103.