THE ROLE OF H-2-LINKED GENES IN HELPER T-CELL FUNCTION

II. Isolation on Antigen-Pulsed Macrophages of Two Separate Populations of F₁ Helper T Cells Each Specific for Antigen and One Set of Parental H-2 Products

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The mechanisms responsible for immune recognition and the control of subsequent effector cell functions are topics immunologists are beginning to investigate at the level of the gene. It appears that the cell-cell interactions required during each of these phases of the immune response are under direct control of genes that map in the major histocompatibility complex (MHC). Thus, the role of the macrophage (Mφ) in the processing and/or presentation of antigen to T and B lymphocytes has been reevaluated to include certain genetic restrictions observed among the interacting cell types. Primed guinea pig T cells, for example, become optimally stimulated to proliferate in vitro only when exposed to antigen-pulsed Mφ sharing identical I region-associated (Ia) histocompatibility antigens. Similar restrictions involving various loci within the H-2 complex of the mouse have been demonstrated for T-B cooperation resulting in in vivo antibody production; Mφ-T cell interactions in in vitro secondary antibody responses; cytotoxic T-cell target recognition elicited by viral-infected or chemically modified cells; successful transfer of delayed type hypersensitivity in vivo; and T-cell suppression of various functions.

A unifying interpretation of these results, tested in our laboratory and a number of others, suggests that T lymphocytes initially recognize antigen together with MHC membrane components on the antigen-presenting cell (dual or associative recognition). In the course of subsequent interactions, the T cell must again "see" this same display of antigen + MHC product(s) in order to proliferate or effectively function.

However, the exact nature of this genetic control, particularly for helper function, remains a controversial issue. Many of the studies in this area have been performed in vivo, where it is difficult to assess the individual contributions of all cell types involved, or have centered on in vitro interactions primarily between Mφ and T cells or unseparated lymphocytes.

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Abbreviations used in this paper: B10, C57BL/10; BDF₁, (C57BL/6 × DBA/2) F₁ hybrid mice; BSS, balanced salt solution; FCS, fetal calf serum; FRBC, frog erythrocytes; HRBC, horse erythrocytes; Ia, I region associated; Ir, immune response; Mφ, macrophage(s); MHC, major histocompatibility complex; PEC, peritoneal exudate cells; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenylated.
In previous experiments, we sought to determine the requirements for effective cell collaboration using defined populations of T cells, B cells, and Mφ in the secondary antibody response in vitro. Our results indicated that in F1 mice, T helper cells primed by antigen-bearing parental Mφ cooperate preferentially in the secondary response with B cells and Mφ of the same parental H-2 type (13). This restriction was controlled at least at the level of the B cell because F1 T cells primed with antigen on Mφ from one parent would not cooperate well with B cells from the other parent even in the presence of the first parent's Mφ (14). From this we theorized that in F1 mice there should exist distinct T-cell populations each capable of recognizing antigen in association with an MHC gene product of one or the other parent. These individual helper cells, once triggered, would then be restricted in their ability to cooperate with B cells bearing the same H-2 products on their surface.

In this paper we report the isolation of antigen-specific helper T cells by selective binding to antigen-pulsed Mφ monolayers. With this technique, we show that sheep erythrocyte (SRBC)-primed F1 mice whose parents differ at H-2, contain two haplotype-specific helper T-cell populations, each able to bind to antigen-pulsed Mφ monolayers, and cooperate preferentially with B cells and Mφ bearing one parental H-2 type. The haplotype preference exhibited by these cells is not the result of suppressive activity directed against the opposite parental H-2 type. Furthermore, the ability of these helper cells to cooperate is restricted at least by the H-2 type of the B cell, because F1 helper T cells isolated on SRBC-pulsed Mφ bearing one of the parental H-2 haplotypes are unable to cooperate with B cells and Mφ bearing the other parental H-2 haplotype even though Mφ of the first H-2 haplotype are added to the cultures.

Materials and Methods

Mice. Female (C57BL/6 x DBA/2) F1 hybrid (BDF1) mice (H-2b x H-2d) and congenic C57BL/10Sn (B10) (H-2b) and B10.D2/nSn (H-2d) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Antigens. SRBC (Bellweather Farms, Palmyra, N. Y.) and horse erythrocytes (HRBC; Colorado Serum Co., Denver, Colo.) were obtained from single animals and stored in Alsever's solution. Trinitrophenylated (TNP) erythrocytes were prepared by the method of Rittenberg and Pratt (15) as modified by Kettman and Dutton (16). Frog (Xenopus laevis) erythrocytes (FRBC) were generously provided by Dr. Nicholas Cohen.

Immunizations. Mice were primed by intravenous injection with 8 × 10^6 SRBC in balanced salt solution (BSS; 17, 18). 4 days later, spleens were removed and used as a source of SRBC-primed helper T cells. In vitro immunizations consisted of 2 × 10^6 SRBC or heavily conjugated TNP-SRBC per milliliter of culture medium.

Preparation of T and B Cells. The preparation of purified splenic T cells on nylon fiber columns has been described in detail (19). T-depleted populations were obtained by treatment of mice in vivo with rabbit anti-mouse thymocyte serum (Microbiological Associates, Bethesda, Md.) combined with anti-T serum + complement treatment of spleen cells in vitro as previously described (20). These preparations were used as a source of B cells and Mφ.

Preparation of Peritoneal Mφ Monolayers. Peritoneal exudate cells (PEC) were induced by intraperitoneal injection of nonimmunized mice with 3 ml of 3% fluid thioglycollate. 5 days later, the peritoneal cavities were washed with cold 5% fetal calf serum (FCS) in BSS containing 10 U/ml heparin, the harvested PEC were washed twice and resuspended in Eagle's Minimal Essential Medium (modified F-14) supplemented with nonessential amino acids (21), sodium pyruvate (21), 0.2 mM glutamine, penicillin and streptomycin, 5% FCS (Microbiological Associates, lot no. 90092), and 5 × 10^-3 M 2-mercaptoethanol (22). This is referred to as complete medium. PEC were plated in Linbro FB16-24TC culture trays (Linbro Chemical Co., New Haven, Conn.) at a concentration of 1 × 10^6/ml per culture well and incubated at 37°C in an atmosphere of 10% CO2,
90% air. After 4-5 h, the nonadherent cells were removed by vigorous trituration of the cultures with a Pasteur pipette followed by three additional washes with warm 5% FCS-BSS. All monolayers were observed with the aid of an inverted microscope for uniform distribution and for the morphologic appearance of typical peritoneal Mφ. Monolayer cultures were then incubated for 18-20 h at 37°C either with no antigen or with 5 × 10⁶ SRBC or FRBC in complete medium.

After this antigen "pulse," the monolayers were washed three times with 5% FCS-BSS to remove free erythrocytes and reincubated in complete medium for an additional 4 h on a rocking platform (Belco Glass, Inc., Vineland, N. J.) to allow for additional release of antigen. At that time, the Mφ were washed before the addition of primed T cells to the monolayer.

Fractionation of Helper T Cells on Mφ Monolayers. Erythrocytes and dead cells were removed from nylon column-purified T cells from SRBC-primed mice by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.; 23). The recovered T cells were washed twice and resuspended in complete medium. Individual wells containing Mφ monolayers received 5 × 10⁶ primed T cells in 0.7 ml. The Mφ-T cell mixtures were then maintained at 37°C on a slowly rocking platform for 20-22 h.

After incubation, the cultures were swirled gently, and unbound lymphocytes were recovered and used as the nonadherent T-cell fraction. The cells removed during three additional gentle washes of the monolayers were discarded. Finally, those T cells strongly adherent to the Mφ monolayer (adherent T-cell fraction) were resuspended by trituration of the cultures with 1.0 ml 5% FCS-BSS. Both adherent and nonadherent fractions were further incubated on small (2.0-ml) nylon fiber columns (19) to remove contaminating Mφ before use in culture.

Culture Conditions. Primary and secondary antibody responses were induced in vitro by modifications of the methods of Mishell and Dutton (19, 21). Triplicate cultures were established in 0.5 ml complete medium each in Linbro FB16-24TC culture trays at concentrations of 4-5 × 10⁶ cells/culture well. Cultures were incubated at 37°C in an atmosphere of 10% CO₂/90% air on a rocking platform and were fed daily with 0.1 vol nutrient cocktail (21). After 4 days, identical wells were pooled and assayed in duplicate or triplicate for direct (IgM) plaque-forming cells.

In some experiments, spleen cell cultures contained additional Mφ prepared from the peritoneal cavities of normal noninduced mice. Individual culture wells received 1 × 10⁶ peritoneal cells in 0.5 ml medium. After overnight incubation, the nonadherent cells were removed by vigorous washing, and spleen cells were cultured on the remaining Mφ.

Plaque-Forming Cell (PFC) Assay. Anti-SRBC and anti-TNP responses were enumerated by the slide modification (21) of the Jerne hemolytic plaque assay (24). Parallel determinations were made on HRBC and lightly conjugated TNP-HRBC, and the difference taken as the number of anti-TNP PFC.

Assay for Primed Helper T-Cell Activity. The helper activity of carrier primed T cells in the in vitro anti-TNP response of normal B cells has been described previously (17). In brief, varying numbers of SRBC-primed helper T cells (fractionated or unfractionated) were added to constant numbers of B cells and Mφ, and the cultures were immunized with free TNP-SRBC antigen. A plot of the resulting anti-TNP PFC per culture vs. the number of helper cells added yielded a titration with an initially linear slope. A least squares line was fitted to these points by computer analysis with simple linear regression, and the slope of this line was taken as the activity of the helper T-cell population.

Results

Isolation of Helper T Cells by Binding to Antigen-Pulsed Mφ Monolayers. In view of the physical interactions observed in vitro between immune guinea pig T cells and antigen-bearing Mφ (25), we predicted that primed murine helper cells might similarly recognize and bind to antigen-pulsed syngeneic Mφ. If the Mφ-adherent T cells could be recovered functionally intact, they should represent a population highly enriched for helper activity to the priming antigen. Our initial efforts, therefore, were to determine conditions under which binding and enrichment of helper T cells could actually be observed.
Carrier-specific T cells were obtained from groups of BDF1 mice primed in vivo with SRBC. Our basic protocol consisted of incubating the primed lymphocytes with antigen (SRBC)-pulsed syngeneic Mφ monolayers, harvesting the Mφ-adherent and nonadherent T-cell fractions, and titrating each for primed helper activity as described in Materials and Methods. In preliminary experiments, we found that short-term incubation of Mφ and T cells (1 1/2 h) failed to result in specific selection of helper cells on the monolayers. Both adherent and nonadherent T cells exhibited the same degree of helper activity as unfractionated cells. Because Lipsky and Rosenthal (25) have shown that a 20-h incubation of T cells with Mφ is required to distinguish between antigen-dependent and antigen-independent Mφ-T cell binding in the guinea pig, we decided to modify our protocol so that SRBC-primed T cells were incubated with antigen-pulsed Mφ for 20 h before adherent and nonadherent T-cell populations were harvested. The results of one such experiment are shown in Fig. 1. The helper activity of the Mφ-adherent T-cell fraction was six to ninefold greater than either the unfractionated (primed T cells left alone in culture dishes for 20 h) or the nonadherent T cells, respectively. Similar enrichment for primed helper activity on antigen-pulsed Mφ has been obtained in nine additional experiments. The T-cell recovery in the Mφ-adherent fraction usually represented only 2–8% of the total applied to the monolayer, whereas 25–35% were recovered as nonadherent T cells. As yet, we have been unable to completely deplete the nonadherent T-cell fraction of helper activity which indicates that not all antigen-reactive T cells are effectively bound to the monolayer under these conditions.

Antigen Specificity of Mφ-Adherent Helper Cells. It remained to be determined whether the apparent enrichment for helper activity in the adherent T-cell fraction occurred because antigen-specific T cells recognized and bound to antigen on the Mφ surfaces or resulted merely from nonspecific stimulation of the T cells caused by cell contact or activated Mφ products. We examined this problem in two ways. SRBC-primed BDF1 T cells were applied to syngeneic Mφ monolayers, half of which were pulsed with SRBC and the other half left unpulsed. After 20 h, the Mφ-adherent and nonadherent T-cell fractions were recovered from each type of monolayer and titrated for helper activity as above. In separate experiments, similarly primed BDF1 T cells were fractionated on SRBC-pulsed Mφ and also on monolayers pulsed with a noncross-reacting antigen, FRBC. The results of both experiments, shown in Fig. 2, indicate that enrichment for SRBC-primed helper activity occurred only in those T cells adherent to Mφ monolayers pulsed with the same antigen to which the T-cell donors had been primed. T cells adherent to monolayers left unpulsed or pulsed with an irrelevant antigen, showed no greater activity than the nonadherent fractions. Each of these experiments was repeated twice with similar results. At present, these data do not enable us to determine whether enrichment occurs solely by binding of antigen-specific T cells to the appropriate antigen-pulsed monolayer or whether additional cell activation also takes place. If activation does occur, however, it is both antigen specific and, as shown in the following section, H-2 restricted.

Isolation of Helper T Cells Recognizing Antigen and H-2. Having established a technique that enabled us to isolate antigen-specific T cells enriched
for helper function, we were able to design experiments to determine whether these T cells are also specific for MHC gene products expressed on the antigen-presenting cell. Nylon-purified T cells from SRBC-primed BDF₁ mice were plated onto SRBC-pulsed monolayers as usual. In these experiments, however, the monolayers were prepared from B10 (H-2b) or B10.D2 (H-2d) mice. These strains represent H-2 congenics of the B10 background carrying one or the other parental H-2 haplotypes found in BDF₁ hybrids.

After a 20-h incubation, the T cells strongly adherent to each type of monolayer were isolated and titrated for their ability to cooperate with B cells and Mφ of either parent in the anti-TNP response. T cell-depleted spleens from the H-2 congenic parents were used as sources of B cells and Mφ in the helper cell assay to avoid allogeneic interactions between parental and F₁ T cells in culture. One of four similar experiments is depicted in Fig. 3. In cultures containing B10 B cells and Mφ (Fig. 3 A), the F₁ T cells isolated on B10 Mφ monolayers were much more efficient helpers than F₁ T cells isolated on B10.D2 monolayers. Conversely, only the adherent F₁ T cells isolated on B10.D2 monolayers could cooperate with B10.D2 B cells and Mφ (Fig. 3 C). Both types of adherent F₁ T cells, however, cooperated efficiently with syngeneic BDF₁ B cells and Mφ (Fig. 3 B). The slightly higher activity seen here with B10.D2-isolated T cells is a consistent finding in our laboratory.

These results strongly suggest that primed F₁ mice contain separate populations of haplotype-specific helper T cells, each able to recognize and bind to antigen-pulsed Mφ monolayers from one parent. The F₁ T cells isolated by
FIG. 2. Enrichment for helper activity is specific for the priming antigen. Purified T cells were obtained from the spleens of groups of seven SRBC-primed BDF₁ mice. In A half of the primed T cells were incubated with SRBC-pulsed BDF₁ Mφ and half with Mφ alone (unpulsed). In B half of the primed T cells were again incubated with SRBC-pulsed BDF₁ Mφ and half with FRBC-pulsed BDF₁ Mφ. After 20 h the adherent and nonadherent T-cell fractions were harvested and titrated for helper activity as in Fig. 1. For each titration, the number of anti-TNP PFC per culture was plotted vs. the number of T cells added, and the initial slope determined by the least squares method. This slope ± SE is shown for each titration and was taken as a measure of the relative activity of primed helper T cells in that population.

FIG. 3. Helper activity of SRBC-primed BDF₁ T cells isolated on B10 and B10.D2 Mφ monolayers: H-2 restriction in the antihapten (TNP) response. T cells were obtained from the spleens of seven to eight SRBC-primed BDF₁ mice. One portion was incubated with SRBC-pulsed B10 Mφ monolayers and another portion with SRBC-pulsed B10.D2 Mφ. After 20 h the T cells adherent to each type of Mφ were isolated and titrated for helper activity using B cells and Mφ from B10 (A), BDF₁ (B), and B10.D2 (C) spleen cells. TNP-SRBC was used as antigen in all cultures. The slopes ± SE of the titration lines are shown.
binding to B10 Mφ are deficient in B10.D2-specific cells, and therefore cooperate effectively only with B10 B cells and Mφ in the helper cell assay. The converse is true for F1 T cells isolated on B10.D2 Mφ monolayers.

Because anti-SRBC responses can be elicited nonspecifically by a number of means (26), we were prompted to determine whether the anticarrier response (anti-SRBC) in our studies exhibited histocompatibility restrictions similar to that of the antihapten response. When the same experiments reported above were assayed for anti-SRBC PFC in addition to anti-TNP, exactly the same types of results were obtained (Fig. 4). F1 T cells isolated on monolayers of one parental haplotype cooperated preferentially in the secondary anti-SRBC response with B cells and Mφ of the identical haplotype. Thus, we concluded that both the antihapten and anticarrier antibody responses are controlled by antigen-specific, H-2-restricted T cells.

It is worth mentioning that in control experiments (not shown here), primed T cells from BDF1 mice isolated on unpulsed parental Mφ monolayers showed no restriction when tested for helper activity with B cells and Mφ from either parent. Apparently, the isolation of H-2-restricted helper cells must in some manner involve the recognition of both antigen and H-2 products on the Mφ.

H-2 Restriction of Helper Cells is Not Due to Suppression. An alternative explanation for the haplotype preference observed in the previous experiments might be that the T cells isolated on Mφ of one parent contained or were induced to contain suppressor cells directed against the other parent. Therefore, rather than being unable to cooperate with that parental haplotype, they might be actively suppressive for responses by it. If this were so, however, we would have expected that when either of the Mφ-isolated F1 T-cell populations was tested with F1 B cells and Mφ for helper activity, they would have recognized the opposite haplotype on the F1 cells and suppressed the response. As was shown in Fig. 3B, however, both T-cell populations cooperated extremely well with F1 B cells and Mφ.

Fig. 4. Helper activity of SRBC-primed BDF1 T cells isolated on B10 and B10.D2 Mφ monolayers: H-2 restriction in the anticarrier (SRBC) response. Same as in Fig. 3 except that the cultures were assayed for anti-SRBC PFC.
Fig. 5. H-2 restriction of haplotype-specific T cells is not due to suppression. SRBC-primed BDF1 T cells were isolated by adherence to B10 and to B10.D2 MΦ monolayers as in Fig. 3. Each of these T-cell populations (hatched bars), as well as a 1:1 mixture of the two (open bars), was titrated for helper activity in the anti-TNP response using B10.D2 B cells and MΦ (A), and B10 B cells and MΦ (B). The slope ± SE is shown for each titration. The dotted line indicates the calculated average of the two responses observed for each population alone.

To further test for the existence of suppressor phenomena, we took two additional experimental approaches. In one, primed F1 T cells isolated on antigen-pulsed B10 and B10.D2 MΦ monolayers were prepared. These populations and a 1:1 mixture of the two were tested for helper activity with each of the parental-type B cells and MΦ. Fig. 5A indicates that F1 T cells isolated on B10.D2 MΦ cooperated well with B10.D2 B cells whereas the T cells isolated on B10 MΦ cooperated poorly. If the B10-isolated T cells were unable to function because of suppressive activity specific for the B10.D2 haplotype, then the helper activity of the 1:1 mixture should be less than the calculated average of the responses observed with the two populations alone. However, there was no significant difference between the observed and the calculated responses. Similar results were also obtained when B10 B cells and MΦ were tested (Fig. 5B).

A second approach was suggested by the observations of Pierce and co-workers (27), who studied the in vitro responses of spleen cells from mice primed with antigen-bearing allogeneic MΦ. These cells could develop secondary PFC responses when presented with antigen bound to the same allogeneic MΦ used in priming but were unable to develop primary responses to antigen bound to syngeneic MΦ. One implication was that a selective suppression of the primary response could occur. Thus, we attempted to determine whether in our system isolated T cells that were H-2 restricted in the secondary response to SRBC could specifically suppress the primary SRBC response of the alternate parental haplotype.

To ensure the absence of allogeneic interactions caused by cell mixing,
TABLE I

Failure of SRBC-Primed T cells Isolated on B10.D2 Mφ to Suppress the Primary SRBC Response of B10 B Cells

<table>
<thead>
<tr>
<th>Unprimed B-cell source</th>
<th>Unprimed BDF,</th>
<th>SRBC-primed BDF, isolated on B10.D2 Mφ</th>
<th>Anti-SRBC PFC/culture*</th>
</tr>
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<tr>
<td>4 x 10⁶ B10 B cells + Mφ</td>
<td>--</td>
<td>--</td>
<td>145</td>
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<tr>
<td>&quot;</td>
<td>6 x 10⁵</td>
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<td>2,530</td>
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<tr>
<td>&quot;</td>
<td>6 x 10⁵</td>
<td>0.2 x 10⁵</td>
<td>2,327</td>
</tr>
<tr>
<td>&quot;</td>
<td>6 x 10⁵</td>
<td>0.4 x 10⁵</td>
<td>2,667</td>
</tr>
<tr>
<td>&quot;</td>
<td>6 x 10⁶</td>
<td>0.6 x 10⁵</td>
<td>2,790</td>
</tr>
<tr>
<td>&quot;</td>
<td>6 x 10⁵</td>
<td>0.8 x 10⁵</td>
<td>2,553</td>
</tr>
<tr>
<td>&quot;</td>
<td>6 x 10⁵</td>
<td>1.0 x 10⁵</td>
<td>2,700</td>
</tr>
<tr>
<td>4 x 10⁶ B10 B cells + Mφ</td>
<td>--</td>
<td>0.2 x 10⁴†</td>
<td>140</td>
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<tr>
<td>&quot;</td>
<td>--</td>
<td>0.4 x 10⁴</td>
<td>207</td>
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<tr>
<td>&quot;</td>
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<td>0.6 x 10⁴</td>
<td>167</td>
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<tr>
<td>&quot;</td>
<td>--</td>
<td>0.8 x 10⁴</td>
<td>460</td>
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<tr>
<td>4 x 10⁶ B10.D2 B cells + Mφ</td>
<td>--</td>
<td>0.2 x 10⁴‡</td>
<td>155</td>
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<tr>
<td>&quot;</td>
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<td>0.4 x 10⁴</td>
<td>338</td>
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<tr>
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<td>--</td>
<td>0.6 x 10⁵</td>
<td>840</td>
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<tr>
<td>&quot;</td>
<td>--</td>
<td>0.8 x 10⁵</td>
<td>1,330</td>
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</table>

* PFC assayed on day 4 from triplicate cultures immunized with SRBC.
† Helper activity of BDF, T cells (B10.D2 Mφ isolated) tested with B10 B cells: 3,647 ± 1,471 PFC/culture per 10⁶ T cells.
‡ Helper activity of BDF, T cells (B10.D2 Mφ isolated) tested with B10.D2 B cells: 27,912 ± 6,334 PFC/culture per 10⁶ T cells.

cultures were constructed to contain T-cell-depleted B10 spleen cells as a source of unprimed B cells and Mφ, and an optimal number of nylon-passed normal BDF, spleen cells as an unprimed helper T-cell source. Under these conditions, the primary response of B10 cells to SRBC after 4 days in culture was 2,530 PFC/culture (Table I, top). SRBC-primed F, T cells isolated on antigen-pulsed B10.D2 Mφ monolayers were also prepared. When these B10.D2 "restricted" helpers were titrated into the above cultures, no decrease in the primary anti-SRBC response was observed (Table I). Control experiments, which were run simultaneously, ensured that the B10.D2-isolated T cells were indeed restricted for helper activity to B10.D2 B cells and not to B10 B cells (Table I, bottom).

Thus, we concluded that F, T cells, which could only cooperate well with the B10.D2 haplotype in the secondary anti-SRBC response, could not actively suppress the primary response of the B10 haplotype to that same antigen.

Restriction of Haplotype-Specific Helper T Cells at the Level of the B Cell. The above experiments demonstrated that primed F, T cells isolated on antigen-pulsed Mφ of a particular parent cooperated in the absence of suppression only with B cells and Mφ of the same parent. The data, however, did not indicate whether the B cells, the Mφ, or both were the actual targets of this haplotype preference. It was possible that the restriction we observed might only have been necessary for efficient Mφ-T cell interaction, and that the H-2 type of the B cells in the helper cell assay was unimportant.
FIG. 6. H-2 restriction of haplotype-specific T cells at the level of the B cell. SRBC-primed T cells were isolated by adherence to B10 and B10.D2 Mφ as in Fig. 3. These T cells were titrated for helper activity in cultures containing B10 B cells and Mφ (A), and B10.D2 B cells and Mφ (B). Some cultures contained 1 × 10^6 additional peritoneal Mφ of the same H-2 type as the Mφ on which the F₁ cells were isolated. The slopes ± SE for each titration in the anti-TNP response are shown.

On the other hand, if H-2 restriction was expressed at least at the level of the B cell, as suggested by our previous work (14), we reasoned that isolated F₁ T cells that were restricted for one of the parental haplotypes would fail to cooperate with B cells of the other parent even if the cultures contained additional Mφ from the first parent. To test this, experiments were performed as described in Fig. 6.

SRBC-primed F₁ T cells were again isolated by adherence to SRBC-pulsed B10 or B10.D2 Mφ monolayers. Both T-cell populations were then tested for helper activity with B cells and Mφ from either parent. As shown in Fig. 6 A, the F₁ T cells isolated on B10 monolayers (B10-restricted) functioned efficiently with B10 B cells and Mφ. F₁ T cells isolated on B10.D2 monolayers (B10.D2-restricted) did not. If B10.D2 Mφ were included in these latter cultures, the B10.D2-restricted T cells remained unable to cooperate with the B10 B cells. The same conclusion was reached after reciprocal experiments testing B10.D2 B cells and Mφ (Fig. 6 B). Both experiments were repeated twice with similar results. These additional Mφ represent the adherent cells from 10^6 noninduced peritoneal cells prepared as described in Materials and Methods. In our hands, such cells constitute a completely functional Mφ population which is capable of restoring the in vitro PFC response of Mφ-depleted T cells and B cells to TNP-SRBC (19). In the above experiments, however, these Mφ were unable to circumvent the requirement for H-2-restricted interactions between T and B cells in culture. In other experiments (not shown), identical results were obtained with antigen-pulsed Mφ added to the appropriate cultures. The SRBC-pulsed Mφ in this case were derived from the same Mφ population originally used to isolate the F₁ T cells.
Therefore, H-2 restriction for helper T-cell function cannot be only at the level of Mϕ-T cell interaction, but must at least be expressed at the B-cell level.

Discussion

In this paper we describe a method for isolating antigen-specific helper T cells based on their ability to bind to antigen-pulsed Mϕ monolayers. SRBC-primed T cells that remained adherent to antigen-pulsed syngeneic Mϕ after 20 h in culture were found to be markedly enriched for helper activity when tested in vitro in the anti-TNP-SRBC response. Successful binding and enrichment was shown to require the presence on the monolayer of the specific antigen to which the T-cell donors had been primed. Although this result tends to rule out any type of nonspecific enhancement of helper function caused by Mϕ-T cell association (28) or activated Mϕ products (29, 30), we have not as yet been able to completely elucidate the mechanism of enrichment. After a 20-h co-culture of T cells and antigen-pulsed Mϕ, we cannot be sure whether the enriched activity present in the Mϕ-adherent fraction is due simply to the physical attachment of specific T cells to Mϕ-bound antigen, or whether some element of specific T-cell activation is also involved. We do know that this method does not completely deplete the nonadherent T-cell population of specific helper activity.

Nevertheless, we were able to use this enrichment technique to study the nature of the genetic control that determines how these T cells interact with antigen and with other cells involved in in vitro antibody synthesis. For this purpose, we isolated SRBC-specific F1 helper T cells on antigen-pulsed Mϕ prepared from H-2 congenic mice bearing either of the parental H-2 haplotypes. The enriched cells were then assayed for helper activity in the in vitro response to TNP-SRBC using B cells and Mϕ prepared from the H-2 congenic strains. Our results demonstrated that SRBC-primed BDF1 T cells that bound to SRBC-pulsed Mϕ of the H-2d haplotype could function as helper cells in vitro with H-2d B cells and Mϕ but not with B cells of the alternate (H-2b) parental haplotype. In similar fashion, primed F1 T cells selected on antigen-pulsed H-2b Mϕ represented a separate population which cooperated preferentially with H-2b B cells and Mϕ. This preference was seen both in the response to the hapten, TNP, and to the carrier SRBC. So far we have not been able to perform the reciprocal of this experiment, i.e., when F1 T cells were incubated with antigen-pulsed Mϕ of one parental H-2 haplotype, and nonadherent fraction was not depleted of T cells that cooperate with B cells and Mϕ of that haplotype. However, this result was expected because, as stated above, this technique is effective only in the enrichment, not the depletion, of antigen-specific T cells.

In several recent investigations describing H-2-restricted phenomena (31-33), care had been taken to show that suppressive mechanisms did not influence the interpretation of the results. Therefore, we performed a number of control experiments to rule out the possibility that the H-2 haplotype preference we observed with F1 T cells isolated on Mϕ bearing one parental H-2 haplotype did not result from alloreactive suppressor activity directed against the "nonpre-
ferred" parental H-2 haplotype. First of all, if suppression were H-2 restricted, and if the H-2-coded products responsible for determining restriction were present on all F1 B cells and Mφ (therefore not allelically excluded), we would have expected each of the isolated T-cell populations to recognize the opposite haplotype on F1 B cells and Mφ and suppress the response. This did not occur because in fact both T-cell populations cooperated effectively with F1 B cells and Mφ. Secondly, H-2-restricted T cells isolated on Mφ bearing one parental H-2 haplotype did not suppress the helper activity of T cells isolated on Mφ bearing the other parental haplotype when the two T-cell populations were mixed together in culture.

A third approach was taken in light of the recent experiments of Pierce et al. (27) which suggested that T cells restricted for one H-2 type in the secondary antibody response might in fact suppress the primary response of another H-2 type. However, our results clearly demonstrated that SRBC-primed F1 T cells restricted for one H-2 haplotype did not suppress the ability of unprimed F1 T cells to cooperate (in the primary anti-SRBC response) with B cells and Mφ bearing the other H-2 haplotype.

We concluded, therefore, that as a consequence of in vivo priming, F1 mice contain individual populations of helper T cells, each population specific for antigen as well as a product(s) of the H-2 gene complex of one of the parents, and that these populations can be separately isolated in vitro by adherence to antigen-pulsed Mφ bearing parental H-2 haplotypes.

Our results do not answer a number of questions about the mechanism of this restriction. For instance, although it seems unlikely, we have not yet excluded the possibility that this simultaneous recognition of antigen and H-2 is performed not by a single T-cell population but rather by separate populations, one recognizing H-2 and the other antigen, both of which are essential in the mechanism of helper function. Our experiments also do not address the question of whether the recognition of H-2 and antigen occurs via a single or via two receptors on the T-cell surface. Finally, we cannot at present assess the relative contribution of the recognition of antigen vs. the recognition of H-2 to the physical attachment vs. the activation of the T cell on the Mφ surface. However, this technique should prove useful in the future in our attempt to answer these questions.

Our results are strikingly similar to those reported by Shevach and Rosenthal and co-workers over the past several years (2, 3, 25, 34, 35). Working with guinea pigs, Ben-Sasson et al. (35, 36) have been able to identify and to select on antigen-pulsed Mφ those T cells that respond to antigen with proliferation. Many of the elements of their technique are similar to ours, including the necessity for antigen on the Mφ and the need for overnight culture to avoid antigen-independent T-cell adherence to Mφ. In a similar set of experiments, Paul et al. (33) also concluded that T cells from F1 guinea pigs, whose parents differed at the MHC, contain separate subpopulations recognizing antigen and the MHC gene products of each parent. The main advantage of the technique we present is that rather than the isolation of proliferative T cells, whose immunologic function is unknown, we were able to isolate functional helper T cells. Moreover, this allowed us to study how the simultaneous recognition of
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antigen and H-2 might be involved in the mechanism of helper T-cell function.

Although there is substantial evidence that T cell-Mφ interactions are controlled by MHC-linked genes (2, 3, 6, 13, 14, 32, 37-39), there is considerable controversy as to whether this type of control plays a significant role in the interaction of helper T cells with B cells. Several studies have indicated that MHC genes expressed in B cells are not important in T cell-B cell interactions (27, 40), yet other studies indicate that they are (4, 5, 31, 41, 42). Therefore, we were interested in determining whether, after isolation of F1 T cells on Mφ of parental H-2 haplotypes, the restriction observed was the result of their subsequent interaction with Mφ, B cells, or both in the in vitro assay for helper activity.

Thus, we attempted to improve the poor activity of F1 T cells isolated on Mφ of one parental H-2 haplotype, but tested in vitro with B cells and Mφ of the other haplotype, by including Mφ of the first haplotype in the cultures. Peritoneal Mφ have been used by a number of investigators (43-45) as sources of functional Mφ for in vitro antibody responses. In our laboratory they are commonly used to restore the in vitro responsiveness of Mφ-depleted T cells to Concanavalin A (26) and, more importantly, to restore the ability of Mφ-depleted T and B cells to respond in culture to TNP-SRBC (19). In the experiments reported here, however, the failure of restricted F1 T cells to cooperate with the nonpreferred B cells was not alleviated by the addition to the cultures of peritoneal Mφ of the appropriate H-2 haplotype.

We interpret these results to support the view that, although antigen-primed helper T cells can simultaneously recognize Mφ-bound antigen and H-2 gene products, an identical type of recognition must also take place on the B-cell surface during T cell-B cell interactions. We should stress that H-2-controlled T cell-Mφ interactions, although not sufficient, may still play an essential role in the in vitro helper assay. Indeed, preliminary experiments in this laboratory using mixtures of Mφ-depleted B cells and antigen-pulsed Mφ would indicate that this may be the case.

This conclusion that H-2 genes expressed in B cells are involved in T cell-B cell interactions is consistent with two previous studies of this laboratory (13, 14, 19). In the first, H-2-restricted helper T cells were induced by in vivo priming of F1 mice with keyhole limpet hemocyanin-pulsed Mφ bearing one or the other parental H-2 haplotypes. When subsequently tested in vitro with B cells and Mφ from either parent, the restricted helper T cells were shown to be unaffected by the presence in the cultures of Mφ of the priming H-2 haplotype.

In the second study, we identified an H-2-linked Ir gene(s) which controlled the response of helper T cells to a cross-reacting determinant(s) present on burro erythrocytes and SRBC. When T cells were prepared from F1 mice of high × low responder parents, a high response was seen only when they were tested in vitro with high responder B cells and Mφ, not with low responder cells. The inability of these low responder B cells to cooperate with the F1 T cells was not alleviated by the addition of high responder Mφ to the cultures.

In preliminary experiments, we have seen the same results with in vitro responses to poly-L-(Tyr, Glu)-poly-d,L-Ala--poly-L-lys.

In summary, both our previous results and those presented here support the
view that helper T cells recognize antigen and H-2 gene products simultaneously during their interaction with either Mφ or B cells and that this recognition plays an essential role in the mechanism of helper T-cell induction and function.

Summary

A method was established for isolating antigen-specific murine helper T cells by selective binding to antigen-pulsed macrophage (Mφ) monolayers. Sheep erythrocyte (SRBC)-primed T cells, which remained strongly adherent to SRBC-pulsed syngeneic Mφ after 20 h in culture, were markedly enriched for helper activity when tested in the in vitro antitrinitrophenol (TNP) response to TNP-SRBC. Successful binding and enrichment occurred only if the Mφ were pulsed with the specific antigen to which the T-cell donors had been primed.

The genetic control governing helper function in this system was then examined by using primed F1 T cells isolated on Mφ monolayers from congenic strains bearing parental H-2 haplotypes. SRBC-primed BDF1 (H-2b X H-2d) T cells, which bound to SRBC-pulsed H-2d Mφ, subsequently functioned as helper cells in cultures containing H-2d B cells and Mφ, but not in those containing H-2b B cells and Mφ. They remained unable to collaborate with B cells of the H-2b haplotype even in the presence of additional H-2d Mφ, indicating that H-2 restriction occurs at least at the level of the B cell. Similarly, primed BDF1 T cells isolated on H-2b Mφ cooperated preferentially with H-2b B cells and Mφ. In both cases, the haplotype preference of the T cell was not due to alloreactive suppressor activity. These results suggest that primed F1 mice contain individual populations of helper T cells, each of which recognize antigen in association with a parental H-2 gene product(s) expressed during both Mφ-T cell and T cell-B cell interactions.

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