ANALYSIS OF HISTOCOMPATIBILITY REQUIREMENTS FOR PROLIFERATIVE AND HELPER T CELL ACTIVITY

T Cell Populations Depleted of Alloreactive Cells by Negative Selection*

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Recent studies have revealed numerous examples of cell-cell interactions that are involved in the immune response. A striking feature of many of these events is the requirement for genetic compatiblity at the major histocompatibility complex (MHC)[1] between the participating cells.

This requirement was initially seen as a need for genetic identity at the MHC locus. This concept lead to a model in which signals were passed between cells by cell interaction structures, one on the cell giving and one on the cell receiving the signal (1–3). Later work demonstrated that the need for genetic identity was not an absolute condition (4), and it now seems clear that many, but perhaps not all, of these requirements are a consequence of the nature of T cell specificity and the need to recognize self MHC as well as the foreign antigen (5, 6).

It would appear that pre-T cells that recognize self MHC are selected in the thymus and develop into the functional population of peripheral T cells. These cells are subjected to a further round of selection when confronted by antigen in some association with a particular MHC gene product (7–10).

The cell-cell interaction between T cell and antigen-presenting cell (APC) has been extensively studied using the proliferation assay in which T cell populations from primed animals proliferate when re-exposed to the priming antigen (11). In this system there is general agreement in guinea pigs, mice, and rats that the specificity of the responding T cell population is determined by two rounds of selection: first, by self MHC (in the thymus), and second, by the subset of self MHC involved in antigen presentation.

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cell(s); B6, C57BL/6; B10, C57BL/Sn; B10.A, B10.A/SgSn; B10.BR, B10.BR/SgSn; B10.D2 B10.D2/Sn; "B cells"; the B cell preparation from which the accessory cells were removed; BDF_1, (B6 × DBA/2J)F_1; C, complement; CFA, complete Freund's adjuvant; DBA/2, DBA/2J; [^{125}I]UdR, [^{125}I]iododeoxyuridine; KLH, keyhole limpet hemocyanin; KT_{12}, trinitrophenylated KLH; LN, lymph node(s); MLR, mixed lymphocyte reaction; MHC, major histocompatibility complex; NAE, negative allogeneic effect(s); OT_{12}, trinitrophenylated ovalbumin; OVA, ovalbumin; PAE, positive allogeneic effect(s); PFC, plaque-forming cell(s); RAMB, rabbit anti-mouse brain; SRBC, sheep erythrocytes; T_P, T proliferative; T_H, T helper; TDL, thoracic duct lymphocyte(s); TNP, trinitrophenyl(ated); Z, β-galactosidase; ZT_4, TNP-Z.

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The specificity of the induction of helper T cells has been less well studied but appears to be determined by the same selection process (see below).

In the T cell-APC interaction it is generally accepted that the genetic restriction is determined by the nature of the T cell recognition structure(s) and the recognition of antigen in some form of association with an MHC gene product on the APC surface. It seems possible, as the name suggests, that the APC has some special role in antigen presentation. On the B cell, however, it has been presumed that the relevant antigen is bound to the immunoglobulin receptors. It is not clear then whether the same model of MHC and antigen recognition can be applied to explain a T cell-B cell genetic restriction.

The interaction between helper T cells and B cells has been examined by many investigators; however, there is conflicting evidence concerning the nature of the genetic restrictions in this interaction. In the early studies it has been shown that effective collaboration was only seen when T cells and B cells were identical at the IA locus (5). In these studies the APC was not considered, and it is not clear whether the restriction was between T and B cell or between T cell and APC. In more recent studies in which these and other questions were taken into account some investigators (12–16) have confirmed the original observation. Other studies, however, have shown that T cells and B cells can collaborate across a histocompatibility barrier (17–23), although still another study has shown that different classes of antibody response have different histocompatibility requirements (24).

A second constraint on the T cell-B cell interaction is the need for the T cell and B cell to recognize antigenic determinants on the same molecule (25). It is presumed that the antigen provides the bridge that brings together the interacting T cell and B cell.

In this study we have made a careful investigation of the conditions for the interaction between T cell and APC for both helper and proliferative responses in the same T cell population and the interaction between helper T cells and B cells. We have used T cell populations depleted of alloreactive cells by filtration through an irradiated F_1 host. In a parallel study (W. H. Shih, P. Matzinger, S. L. Swain, and R. W. Dutton. Analysis of histocompatibility requirements for proliferative and helper T cell activity: F_1 cells primed in a parental environment. Manuscript in preparation.) In a parallel study we have used F_1 T cell populations positively selected by priming to antigen in a parental environment.

In both models we have found an absolute requirement for genetic compatibility in the T cell-APC interactions and in both models no evidence for any such requirement in the T cell-B cell interaction has been found. The interaction between T cell and B cell requires the linked recognition of carrier and hapten determinants.

Materials and Methods

Mice. C57BL/6J (B6), C57BL/10Sn (B10), B10.A/SgSn (B10.A), B10.D2/nSn (B10.D2), B10.BR/SgSn (B10.BR), BALB/c, DBA/2J (DBA/2), and (B6 × DBA/2)F_1 (BDF_1) mice were either obtained from The Jackson Laboratory, Bar Harbor, Maine, bred at The Salk Institute for Biological Studies, San Diego, Calif., or bred in our colony at the University of California, San Diego, La Jolla, Calif. B6 mice purchased from the L. C. Strong Research Foundation, Del Mar, Calif. were also used in some experiments. All mice were raised on standard laboratory chow and chlorinated water ad lib. and used at >8 wk of age, except mice used for cannulation, which were sometimes as young as 6 wk.

Antigens. β-galactosidase (Z; Worthington Biochemicals, Freehold, N. J.), keyhole limpet hemocyanin (KLH; Pacific Bio-Marine Laboratories Inc., Venice, Calif.), and ovalbumin (OVA; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) were used as carrier antigens. Trinitrophenylated (TNP)-Z (ZT_4; 4 mol TNP/100,000 dalton), TNP-KLH (KT_8; 8 mol TNP/100,000 dalton), and TNP-OVA (OT_{12}; 12 mol TNP/100,000 dalton) were prepared by the method of Rittenburg and Pratt (26).

Immunizations. Mice used as donors of TNP-primed B cells received mutiple injections of ZT_4, OT_{12}, or KT_8 in aluminum hydroxide gel (Accurate Chemical & Scientific Corp., Hicksville, N. Y.). Mice used for the preparation of carrier-primed lymph node (LN) cells were immunized with 50 μg of OVA or Z in complete Freund's adjuvant (CFA; that contained 1 mg/ml heat-killed *Mycobacterium tuberculosis*, H37Ra strain; Difco Laboratories, Detroit, Mich.) at the base of the tail 6–8 d before being used (27). Mice used to prepare carrier-primed spleen cells were given 100 μg of KLH emusified in CFA in four foot pads followed by two to four intravenous injections of 50 μg of soluble KLH in balanced salt solution at 6- to 12-wk intervals. 3 wk after the last injection, mice were killed for the preparation of spleen cells.

Culture Media. RPMI-1640 was supplemented with 5% heat-inactivated (56°C for 45 min) fetal calf serum (FCS) (Grand Island Biological Company, Grand Island, N. Y.), 10 mM glutamine, 100 μ/ml of penicillin, 50 μ/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

Preparation of Cells

T CELLS. Carrier-primed LN cells were prepared from inguinal and periaortic LN of OVA- or Z-primed mice. LN T cells were obtained by filtration of LN cell suspensions through recycled nylon-wool columns. LN T cells or thoracic duct lymphocytes (TDL) were treated at a concentration of 1×10^7–2×10^7 cells/ml with 25 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min.

"B CELLS". B cells and acessory cells were prepared from TNP-primed spleen cells treated with rabbit anti-mouse brain (RAMB) or anti-Thy-1.2 and complement (C). We have used the designation "B cells" to indicate the B cell preparation from which the accessory cells were not removed.

B CELLS. Adherent cells were removed from TNP-primed spleen cells by sequential passage through two Sephadex G-10 columns. The Sephadex G-10-passed spleen cells were then treated with either RAMB or anti-Thy-1.2 and C.

(APC). Plastic-adherent, antigen-pulsed or unpulsed, overnight-cultured, and RAMB or anti-Thy-1.2 + mitomycin-C-treated APC were prepared from spleens of unimmunized mice.

TDL, Negatively Selected against Allogeneic H-2 Haplotypes. 0.6 ml cell suspension that contained 8×10^7–12×10^7 carrier-primed LN cells or spleen cells of one H-2 type was injected intravenously into H-2-disparate mice that had received 900 rad from a ^{60}Co source 6–8 h before the injection. Thoracic duct fistulas were formed, and cannulas were inserted in the irradiated recipients. Thoracic duct lymph was collected between 18 and 40 h after the injection of cells as described by Sprent (28). The TDL were washed three times with Hanks' balanced salt solution supplemented with 100 μ/ml penicillin and 50 μ/ml streptomycin. The effectiveness of the removal of alloreactivity was tested by mixed lymphocyte reaction (MLR), negative allogeneic effect (NAE) (29), and positive allogeneic effect (PAE) (30).

Culture Conditions, Plaque-forming cell (PFC) Assay, and Proliferation Assay. Lymphoid cells were cultured in Falcon microtest II plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) as previously described (29). Various numbers of T cells, B cells, and APC (as indicated in the figure legends) were cultured for 5 d in the presence of antigen. The number of direct and indirect anti TNP-PFC was determined by a modified hemolytic plaque assay. Cell proliferation was determined on day 4 by the uptake of [^{125}I]iododeoxyuridine ([^{125}I]UdR) present during the last 18 h of culture.

In Vivo Measurement of Cell Collaboration. Various combinations of T cells, "B cells", and antigen-pulsed or unpulsed APC were injected intravenously into irradiated (750 rad, 4–6 h earlier) F_1 mice. Some animals received 4 μg of soluble antigen, others received antigen-pulsed APC. 7 d later, the mice were killed and the numbers of direct (IgM) and indirect (IgM + IgG) anti-TNP-PFC were determined.

Results

Demonstration of Alloreactive Cells in Carrier-primed LN T Cell Populations. Various numbers of OVA-primed, mitomycin-C-treated LN T cells from B10.A mice were cultured with TNP-primed syngeneic B10.A "B cells" (i.e., a B cell preparation from which the accessory cells had not been removed; Materials and Methods) or allogeneic BDF_1 "B cells". The responses to a homologous antigen, OT_{12} (hapten coupled to the carrier for T cell priming), or a heterologous antigen, ZT_4, were measured (Fig. 1). The T cells elicited an antigen-specific response with syngeneic "B cells" and a non-antigen-specific PAE with allogeneic "B cells". At high T cell densities, the allogeneic responses were suppressed, presumably a manifestation of an NAE. The response in the absence of OT_{12} or ZT_4 was essentially the same as when "B cells" were cultured alone. The PAE was thus not mediated by polyclonal activation of allogeneic B cells and took place only in the presence of the TNP-antigen.

MHC Restriction in the Proliferative Response of Carrier-primed T Cells Stimulated by Antigen-pulsed APC. Carrier-primed LN cells were filtered through heavily irradiated (900 rad) allogeneic recipient mice (Fig. 2) to remove alloreactive T cells. 8×10^7–12×10^7 carrier-primed LN cells from strain X were injected intravenously into irradiated strain Y mice. Recipient mice were cannulated the next day, and TDL were collected between 18 and 40 h after cell transfer. The TDL collected during this negative selection period have been shown to consist almost entirely (>90%) of donor-derived T cells that are negative in MLR against the recipient, Y (28). The draining TDL were divided into two populations: T cells for the proliferative assay, and T cells for the humoral response assay. For the proliferative assay, washed, untreated X_{-Y} (strain

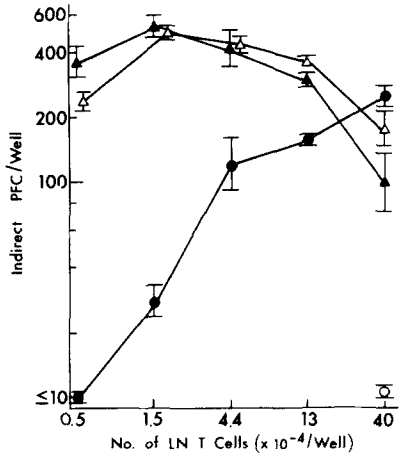

FIG. 1. Unselected carrier-primed LN T cells elicit an antigen-specific response with syngeneic B cells and an antigen-non-specific response with allogeneic B cells. B10.A mice were primed with 50 μg of OVA. 7 d later, T cells were prepared from inguinal and periaortic LN by filtration through nylon-wool columns and mitomycin C treatment. Various numbers of T cells were then added to cultures that contained 100 μg/ml of soluble OT_{12}, or ZT_4. In addition, cultures contained 1×10^6 TNP-primed, RAMB-treated spleen cells from B10.A or BDF_1 mice. B10.A + OT_{12} (●), B10.A + ZT_4 (○), BDF_1 + OT_{12} (▲), or BDF_1 + ZT_4 (△). The indirect anti-TNP PFC per culture well was determined on day 5. The geometric means and SE of triplicate culture wells are plotted vs. the numbers of B10.A OVA-primed T cells added per culture well. The control responses in the absence of OT, or ZT, or T cells were <16 FPC/per well.

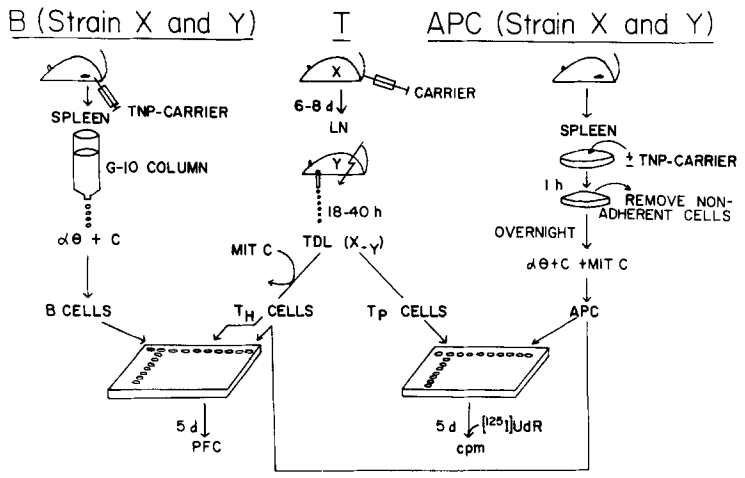

FIG. 2. Protocol for studying the histocompatibility and associative recognition requirements for humoral response. See Materials and Methods and Results for a detailed description.

"X" cells depleted of cells reactive with strain "Y" [e.g., B10.BR$_{-B6}$]) TDL were cultured with antigen-pulsed or unpulsed APC prepared from strain X or strain Y mice. After 4 d in culture, cells were given 0.1 μCi of [^{125}I]UdR. 18–24 h later, the [^{125}I]UdR incorporation in each culture well was measured. APC were prepared from unimmunized spleen cells. After antigen pulsing and adherence in plastic culture dishes, the nonadherent cells were removed. The antigen-pulsed adherent cells were cultured overnight to reduce the amount of subsequent antigen leakage from pulsed APC. The APC were then treated with RAMB or anti-Thy-1.2 + C + mitomycin C to eliminate alloantigen-responding T cells from the population. T cells for the humoral response assay were utilized as described below.

To test for responses in the absence of allogeneic effects, Z-primed B10.BR LN cells were filtered through irradiated B6 mice. The collected B10.BR$_{-B6}$ TDL were cultured with unpulsed syngeneic B10.BR or allogeneic B6 APC to determine the amount of proliferation due to alloreactivity (Fig. 3a). Almost no anti-B6 proliferative activity was observed, indicating that the negative selection had removed the alloreactive T cells. Antigen-specific proliferation of B10.BR$_{-B6}$ TDL was induced by antigen-pulsed syngeneic B10.BR-ZT$_4$-pulsed APC, but not by allogeneic B6-ZT$_4$-pulsed APC (Fig. 3a). The possibility that the requirement for H-2 matching was the result of an otherwise undetectable negative alloreactivity was excluded by the result shown in Fig. 3b. TDL were cultured with a 1:1 mixture of syngeneic and allogeneic APC, restricting the antigen presentation to one or other of the two APC populations. Only when antigen was presented by syngeneic APC was T proliferation induced.

It was found that negatively selected TDL were relatively depleted of APC. Results from four experiments showed that in the presence of an optimal dose of soluble antigen (100 μg/ml), TDL cultured alone gave proliferative response of 25–35% of the cultures in which unpulsed APC were added (data not shown).

Thus in our hands antigen-specific T cells can be negatively selected to yield a population that is completely H-2 restricted in a proliferative assay. We next examined the role of MHC gene products in T helper function.

H-2 Restriction in the Humoral Response. An aliquot of the same preparation of Z-

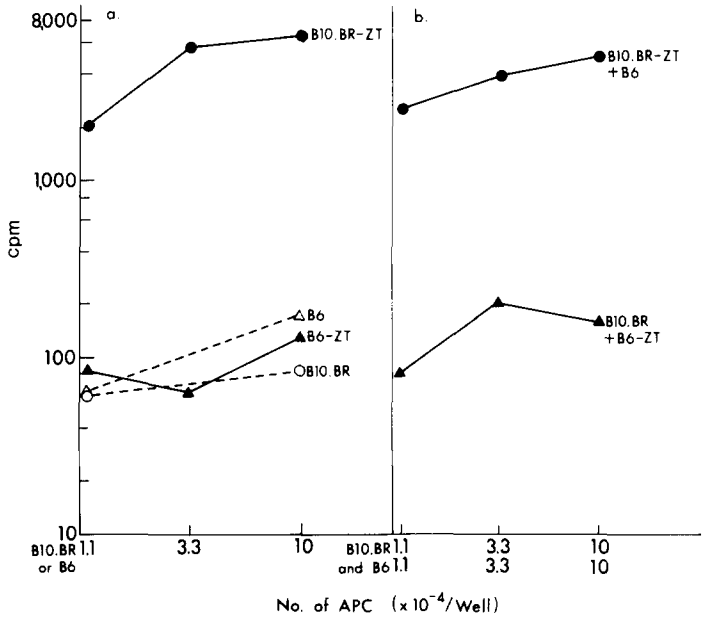

FIG. 3. Antigen-pulsed syngeneic APC elicit an antigen-specific proliferative response in carrier-primed LN T cells, whereas allogeneic APC do not. Z-primed B10.BR LN cells were negatively selected against the B6 haplotype. ZT_4-pulsed and unpulsed APC from B6 and B10.BR mice were prepared as detailed in Materials and Methods and Fig. 2. (a) Various numbers of ZT_4-primed B10.BR (●), ZT_4-primed B6 (▲), B10.BR (○), or B6 (△) APC; or (b) various numbers of 1:1 mixtures of ZT_4-primed B10.BR and B6 (●), or B10.BR and B6-ZT (▲) APC were added to cultures that contained 2.5×10^5 $B10.BR_{-B6}$ TDL. The geometric means of the day-5 [^{125}I]UdR incorporation of triplicate wells of each point were calculated and are plotted vs. the numbers of APC present in each well.

primed $B10.BR_{-B6}$ TDL used above was treated with mitomycin C (to minimize any remaining alloreactive suppressive activity) and tested for helper activity (Fig. 4). $B10.BR_{-B6}$ TDL collaborated with syngeneic B10.BR "B cells" plus APC and not with allogeneic B6 "B cells" plus APC (Fig. 4a). Addition of $B10.BR_{-B6}$ TDLs to cultures containing ZT_4-primed spleen cells from B6 or B10.BR mice did not suppress the responses (Fig. 4b), showing that the lack of collaboration with allogeneic cells was not a result of suppression. Thus T helper function appeared to be H-2 restricted. However, it is not clear from such an experiment where the restriction lies. It could be a result of a requirement for syngenecity between T and B cells, or between T and APC, or both.

Syngeneity between T Cell-APC Level is Sufficient. If the restriction seen above is only required for the T cell-APC interaction, the addition of syngeneic antigen-pulsed APC should allow collaboration with allogeneic B cells. Z-primed $BALB/c_{-B6}$ TDL and allogeneic B6 "B cells" were cultured with ZT_4-pulsed APC from BALB/c or B6 mice (Fig. 5). As the previous experiment (Fig. 4a) had demonstrated, the cultures in which both "B cells" and antigen-pulsed APC were allogeneic, no PFC response was induced. In contrast, T cells cultured with syngeneic BALB/c APC were able to collaborate well with allogeneic B cells. It can be seen that this was not a result of a nonspecific allogeneic effect in that no response was seen with any combination of T, B, and APC and a carrier antigen to which the T cells were not primed (OT_{12}).

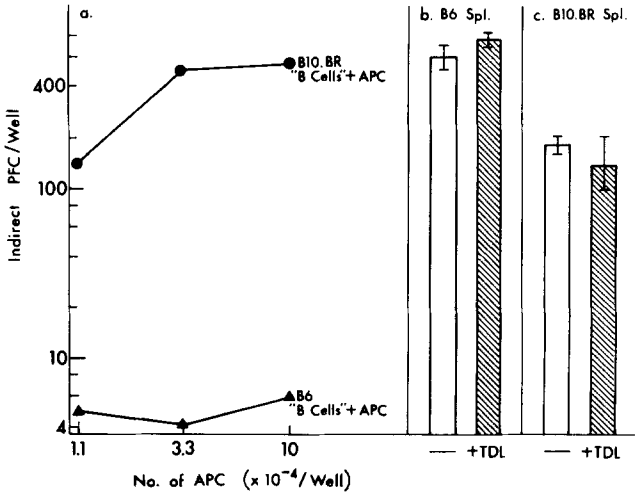

FIG. 4. T_H cells collaborate with syngeneic "B cells" plus APC, not with allogeneic "B cells" plus APC. Cell preparations for APC and TDL were the same as described in the legend of Fig. 3 except that the TDL were treated with mitomycin C and tested for the helper activity. (a) 2.5×10^5 B10.BR$_{-B6}$ TDL were cultured either with 1×10^6 B10.BR "B cells" (RAMB + C-treated, ZT_4-primed spleen cells) and various numbers of ZT_4-pulsed B10.BR APC (●), or with 1×10^6 B6 "B cells" and various numbers of ZT_4-pulsed B6 APC (▲). The responses with the unpulsed APC were <6 PFC/per well. For the NAE control, 2.5×10^5 B10.BR$_{-B6}$ TDL were added to cultures that contained 100 μg/ml of soluble ZT_4, and 1×10^6 ZT_4-primed spleen cells either from B6 (b) or from B10.BR (c).

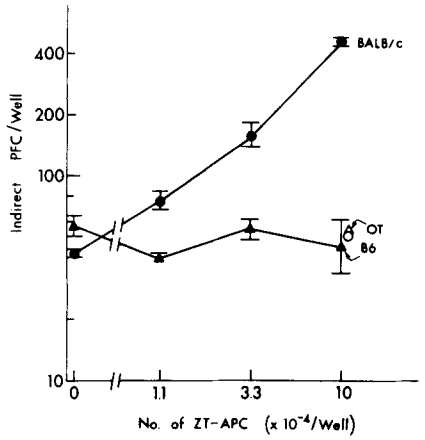

FIG. 5. In the presence of syngeneic APC, helper T cells can collaborate across the histocompatibility barrier with allogeneic B cells. 3×10^5 BALB/c$_{-B6}$ TDL and 1×10^6 B6 "B cells" (RAMB + C-treated, KT_8-primed spleen cells) were cultured with ZT_4-pulsed APC from BALB/c (●) or B6 (▲). To test for a PAE, TDL and B cells were cultured with antigen unpulsed APC from BALB/c (○) or B6 (△) in the presence of 100 μg/ml of soluble OT. The antigen-specific proliferation of TDL required antigen presentation of BALB/c APC and the MLR against B6 was negative.

Hence, the collaboration between T and allogeneic B cells in the presence of antigen-pulsed syngeneic APC's was an antigen-specific response.

The Restriction Requirements Found for LN T Cells also Apply to T Cells Prepared from Spleen. Spleen cells obtained from B6 mice that had received multiple injections of

KLH were also used to prepare negatively selected TDL (Fig. 6). Both T proliferative (T_P) and T helper (T_H) activities were examined and the T cells were also monitored for MLR, and for PAE and NAE. $B6_{-B10.A}$ TDL cultured with allogeneic unpulsed B10.A APC gave a response slightly higher than with syngeneic B6 APC (Fig. 6a). This is the only experiment in which a small residual MLR activity was observed. TDL were cultured with 1:1 mixtures of B6 and B10.A APC, with antigen presented by one of the population (Fig. 6b). It can be seen that only cultures that contained syngeneic KT_8-pulsed B6 APC were stimulated to make an antigen-specfic proliferative response.

To determine the H-2 matching requirement for splenic helper T cells, the same $B6_{-B10.A}$ TDL were mitomycin C treated and cultured with syngeneic B6 "B cells" or allogeneic B10.A "B cells" and KT_8-pulsed or unpulsed APC from B10.A or B6 (Fig. 7). Syngeneic KT_8-pulsed B6 and allogeneic KT_8-pulsed B10.A both stimulated the PFC response when tested with B6 "B cells" (Fig. 7a).

The response with allogeneic pulsed APC illustrated in Fig. 7a was most likely a result of antigen transfer from pulsed KT-pulsed B6 APC to the unpulsed B10.A APC present in the "B cell" population (see below).

The results illustrated in Fig. 7b show that T-B matching is not required. T-APC matching can activate the secondary in vitro anti-TNP response in the absence of T-B matching. Cultures that contained $B6_{-B10.A}$ TDL, allogeneic B10.A "B cells", and KT_8-pulsed B6 APC responded; whereas cultures that contained pulsed allogeneic KT_8-pulsed B10.A APC did not.

PFC Responses with Sephadex G-10-passed B Cells. Preliminary experiments (data not shown) indicated that a single passage through Sephadex G-10 columns did not deplete all APC activity. TNP-primed spleen cells were passed sequentially through two Sephadex G-10 columns before treatment with anti-T cell serum + C (Fig. 8). The two Sephadex G-10 passages reduced the response to 20–30% of that given by cultures restored with additional APC. $B10.A_{-B6}$ TDL (which contain very few APC)

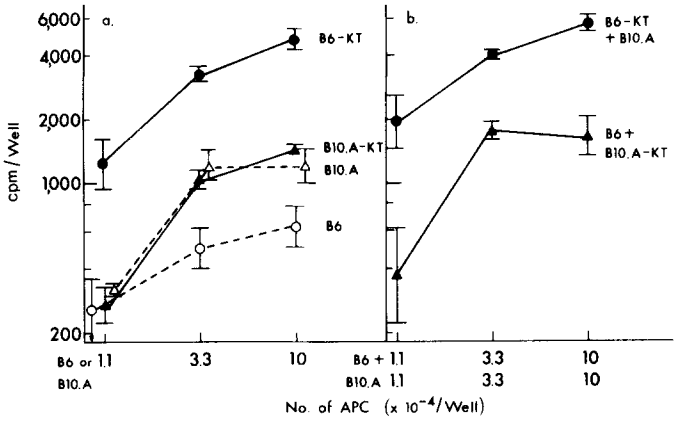

FIG. 6. Antigen-pulsed syngeneic APC elicit an antigen-specific proliferative response in splenic T cells, whereas allogeneic APC do not. KLH-primed B6 spleen cells were negatively selected against the B10.A haplotype. (a) 3×10^5 $B6_{-B10.A}$ TDL were cultured with various numbers of KT_8-pulsed or unpulsed APC from B6 or B10.A mice: KT_8-pulsed B6 (●), KT_8-pulsed B10.A (▲), B6 (○), or B10.A (△) APC. (b) Various numbers of 1:1 mixtures of KT_8-pulsed B6 plus unpulsed B10.A (●), or B10.A-KT plus unpulsed B6 (▲) APC were added to 3×10^5 $B6_{-B10.A}$ TDL.

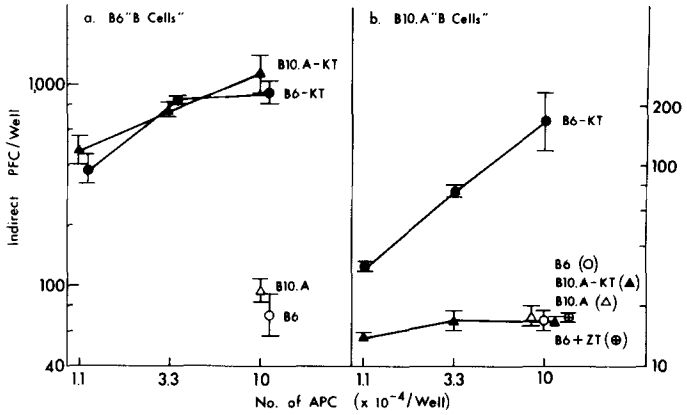

FIG. 7. In the presence of syngeneic APC, carrier-primed splenic T cells collaborate with either syngeneic or allogeneic B cells. Preparations for TDL and APC were the same as described in Fig. 6 except that the TDL were also treated with mitomycin C. 3 × 10^5 $B6_{-B10.A}$ TDL were cultured with B6 (a) or B10.A (b) B plus accessory cells (anti-Thy-1 + C-treated, TNP-primed spleen cells), and various numbers of KT-pulsed or unpulsed APC from B10.A or B6: KT_8-pulsed B10.A (▲), KT_8-pulsed B6 (●), KT_8-pulsed B10.A (△), or B6 (○) APC. For the positive allogeneic control, 3 × 10^5 $B6_{-B10.A}$ TDL and 1 × 10^6 B10.A "B cells" were cultured with 1 × 10^5 B6 unpulsed APC in the presence of 100 μg/ml of soluble ZT_4 (b, ⊕). For negative allogeneic control, 1 × 10^6 ZT_4-primed B10.A spleen cells and 100 μg/ml ZT_4 were cultured with our without the addition of 3 × 10^5 $B6_{-B10.A}$ TDL; the means of indirect PFC responses were 170 and 174, respectively. Similarly, 1 × 10^6 KT_8-primed B6 spleen cells and KT antigen were cultured with or without the addition of TDL, and the responses were 640 and 990, respectively.

were cultured with syngeneic twice Sephadex G-10-passed B10.A B cells and antigen (KT_8) -pulsed B10.A or B6 APC. Allogeneic KT_8-pulsed B6 APC did not allow a response, whereas syngeneic KT_8-pulsed B10.A APC did. Thus the presence of B cells syngeneic to the T cells was not sufficient to generate a response in the absence of syngeneic APC. Similar results were obtained in cultures that contained allogeneic B cells, showing again the syngenecity at the T-APC level is sufficient. In addition to controls that were negative for B10.A anti-B6 MLR, PAE, and NAE (data not shown), we tested whether the presence of allogeneic APC would interfere with an ongoing PFC response. Unpulsed B6 or KT_8-pulsed B6 APC were added to cultures that contained $B10.A_{-B6}$ TDL, B6 B cells, and KT_8-pulsed B10.A APC. The responses were not altered significantly (Fig. 8, legend). We conclude that the PFC response requires syngeneity at T-APC cell level, and that the early observation showing that syngeneic B cells could substitute for syngenecity with APC was misleading. In the absence of contaminating APC, B cells syngeneic to the T cells can not function in the cultures that contain allogeneic antigen-pulsed APC.

Activation of Syngeneic and Allogeneic B Cells by T_H Cells Requires Associative Recognition of Hapten-Carrier Determinants. To test whether the activation of allogeneic B cells has the same antigen recognition requirement as syngeneic cooperation, Z-primed $B10_{-B10.D2}$ TDL were cultured with B cells prepared from syngeneic B6 (Fig. 9a) or allogeneic B10.D2 (Fig. 9b) mice. Cultures also contained one of the following preparations of B6 APC: (*a*) linked antigen (ZT_4)-pulsed, (*b*) non-linked antigens (Z + OT_{12})-pulsed, (*c*) TNP-heterologous carrier antigen (OT_{12})-pulsed, and (*d*) unpulsed APC. Responses with syngeneic or allogeneic "B cells" showed identical patterns. ZT_4-pulsed APC stimulated the PFC responses, whereas OT_{12}-pulsed APC did not.

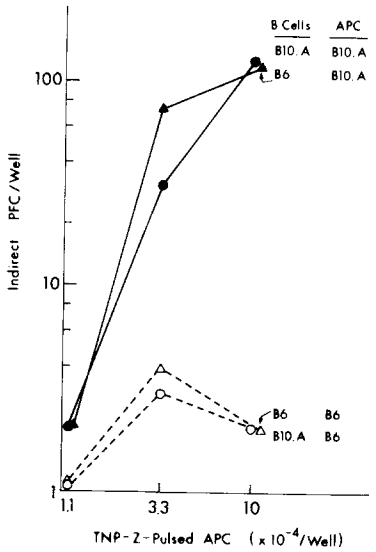

FIG. 8. Histocompatibility requirement for the humoral response. H-2 matching is only required for T-APC interactions, not for T-B or B-APC interactions. B cells were depleted of adherent cells by passing KT_8-primed spleen cells through Sephadex G-10 columns twice before the treatment with RAMB + C. 3×10^5 $B10.A_{-B6}$ TDL were cultured with 1×10^6 B cells and various numbers of ZT_4-pulsed APC from B10.A or B6: B10.A B cells and ZT_4-pulsed B10.A APC (●), B10.A B cells and ZT_4-pulsed B6 APC (○), B6 B cells and ZT_4-pulsed B10.A APC (▲), B6 B cells and ZT_4-pulsed B6-ZT APC (△). Controls to test for B10.A anti-B6 MLR, PAE were included in the experiment and the results were negative. The addition of 1×10^5 ZT_4-pulsed B6 APC or unpulsed B6 APC to cultures that contained 3×10^5 $B10.A_{-B6}$ TDL 1×10^6 B6 B cells, and 1×10^5 ZT_4-pulsed B10.A APC gave PFC responses of 107 and 123, respectively.

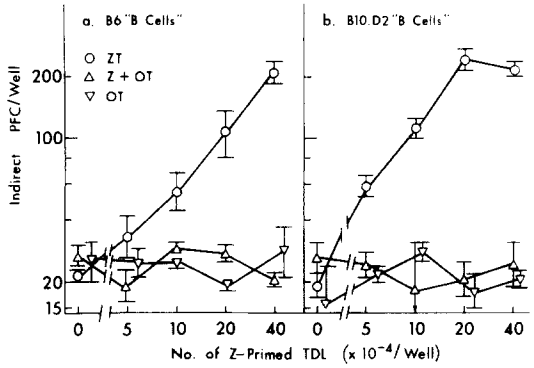

FIG. 9. Associative recognition is required for the interaction between carrier-primed T cells and either syngeneic or allogeneic TNP-primed B cells with Z as the priming carrier antigen, and OVA as the heterologous carrier antigen. Various numbers of mitomycin C-treated, Z-primed $B10_{-B10.D2}$ TDL were added to cultures that contained 1×10^6 (a) or B10.D2 (b) "B cells" (anti-Thy-1 + C-treated, KT_8-primed spleen cells). Cultures also contained 1×10^5 antigen-pulsed B10 APC: ZT_4- (○), Z + OT_{12}- (△), or OT_{12}- (▽) pulsed APC.

The non-linked antigen (Z + OT_{12})-pulsed APC also failed to induce PFC responses. Thus it can be seen that carrier-hapten linkage is required for allogeneic T-B collaboration just as for syngeneic T-B cooperation. Another experiment was carried out using OVA as the priming antigen and Z as the heterologous carrier antigen (Fig.

10). PFC responses were observed in cultures that contained B10.A$_{-B6}$ TDL, "B cells" from syngeneic B10.A, or allogeneic B6 and linked antigen (OT_{12})-pulsed B10.A APC. B10.A APC pulsed with non-linked antigen (OVA + ZT_4) or heterologous antigen (ZT_4) did not stimulate PFC responses. The same results were observed in three other experiments (data not shown).

H-2 Matching Requirements in the In Vivo Measurement of Cell Collaboration. Carrier-primed T cells, TNP-primed "B cells", and antigen-pulsed or unpulsed APC were transferred intravenously into irradiated F_1 hybrid mice. 7 d later, the numbers of direct and indirect anti-TNP PFC/spleen were assayed. In vivo PFC responses are reported as indirect minus direct because the induction of a small primary direct (IgM) anti-TNP PFC response was observed in vivo. In Table I it can be seen that PFC responses were not found in mice that had received Z-primed, negatively selected TDL along with allogeneic "B cells", and allogeneic ZT-pulsed APC from B10.A mice (experiment I) or B10.D2 (experiment II) mice. In contrast, transfer of syngeneic B6-ZT APC promoted good responses from mixtures of T and syngeneic B or from T and allogeneic B cells. Although the positive response of B10.A B cells mixed with ZT-pulsed B6 APC (experiment I) is lower than the response of B6 "B cells", it is significantly higher than the B10.A response seen with the other APC preparations. The lower than the response of B6 "B cells", it is significantly higher than the B10.A responses seen with the other APC preparations. The lower response obtained with B10.A "B cells" compared with B6 "B cells" is most likely a result of differences in the priming of "B cell" preparations. The KT_8-primed B10.A spleen cells from which B10.A "B cells" were prepared gave a lower PFC response than B6 spleen cells (Table I, footnotes g and j). An important feature of the reconstituted B10.A response was that it was ZT_8 specific. Injection of OT_{12}-pulsed B6 APC did not induce anti-TNP IgG PFC responses.

In vivo, as in vitro, T-APC interactions require H-2 matching, and T-B interactions do not.

Associative Recognition Requirements in the In Vivo Response. T cells primed with Z were able to collaborate with either syngeneic or allogeneic "B cells" only in the presence

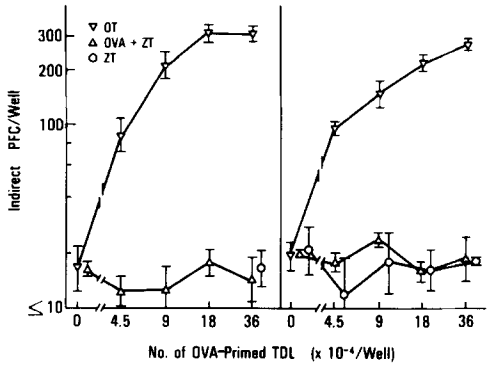

FIG. 10. Associative recognition is required for the interactions between carrier-primed T cells and either syngeneic or allogeneic TNP-primed B cells with OVA as the priming carrier antigen, and Z as the heterologous carrier antigen. Various numbers of mitomycin C-treated, OVA-primed B10.A$_{-B6}$ TDL were added to cultures that contained 1×10^6 B10.A (a) or B6 (b) "B cells" (anti-Thy-1 + C-treated, TNP-primed spleen cells). Cultures also contained 1×10^5 antigen-pulsed B10.A APC: OT_{12}- (▽), OVA + ZT_4- (△), or ZT_4- (○) pulsed APC.

TABLE I

In Vivo Measurement of Cell Collaboration

Experiment	T cells	T. cells primed with	"B Cells"[a]	APC[b]					
				B-ZT_4[c]	B-(Z + OT_{12})	B-OT_{12}	B	A-ZT_4[c]	A
1	Recipient: (B10 × B10.A)F_1[d]								
	3×10^6 $B6_{-B10.A}$ TDL	Z	B6[g]	7640[h]	280	ND[i]	−60	ND	ND
	3×10^6 $B6_{-B10.A}$ TDL	Z	B10.A[j]	1220	−180	20	−60	−60	−220
	3×10^6 B6 LN T Cells	OVA	B6	ND	23,500	18,480	ND	ND	ND
				B-ZT_4	B-(Z + OT_{12})	B-OT_{12}	B	D-ZT[k]	
2	Recipient: (BD)F_1[d]								
	2×10^6 $B6_{-H-2^d}$ TDL's[e]	Z	B6[k]	7430	100	570	−30	ND	
	2×10^6 $B6_{-H-2^d}$ TDL's[e]	Z	B10.D2[l]	5210,4440	−680[m],630[m]	240	−230	230[m]	
	3×10^6 B6 LN Cells[f]	OVA	B6	ND	2410	2750	−30	ND	
	3×10^6 B6 LN Cells[f]	Z	B6	940	ND	−90	−60	ND	

In vivo measurement of cell collaboration verified the in vitro result that associative recognition is required for the interactions between carrier-primed T cells and either syngeneic or allogeneic hapten-primed B cells.

[a] 1×10^7 "B cells" were transferred. "B cells" = anti-Thy-1.2 + C-treated, TNP-primed spleen cells.

[b] 4×10^6 antigen-pulsed or unpulsed APC were transferred.

[c] A, B10.A; B, B6; and D, B10.D2.

[d] F_1 recipient mice were irradiated (750 rad) 4–6 h before cell transfer.

[e] TDL were $B6_{-(B10.D2 + DBA/2)}$.

[f] OVA- or Z-primed B6 LN cells were filtered through nylon-wool columns. However, the yields of nonadherent cells were 90% instead of the usual 30%.

[g] 4 μg of KT_8 and 2×10^7 KT_8-primed B6 spleen cells from which "B cells" were prepared gave PFC response of 73,4000.

[h] Anti-TNP direct and indirect PFC/spleen were determined 7 d after cell transfer. The IgG (indirect-direct) PFC/spleen are reported.

[i] ND, not determined.

[j] 4 μg of KT_8 and 2×10^7 KT_8-primed B10.A spleen cells gave a response of 38,480.

[k] 4 μg of KT_8 and 2×10^7 KT_8-primed B6 spleen cells induced a PFC response of 7,440. TNP-primed spleen cells were treated sequentially twice with anti-Thy-1.2 + C to ensure the complete elimination of T cell activity. The responses of "B cells" alone with priming antigens were −60 for B6 cells and −200 for B10.D2 cells.

[l] 4×10^6 ZT_4-pulsed B10.D2 APC and 2×10^7 ZT_4-primed B10.D2 spleen cells induced 19,860 PFC.

[m] Almost entirely a primary response to OT because most the plaques are IgM.

of syngeneic APC that had been pulsed with ZT_4 (Table I). APC pulsed with Z + OT_{12} were unable to promote a response. To show that OT_{12}-pulsed APC were functionally competent, a response with OT_{12}-primed T cells was included. OVA-primed B6 LN T cells or LN cells were transferred with B6 "B cells" and Z + OT- or OT-pulsed B6 APC. Comparable responses were induced. Thus, pulsing APC with two different antigens did not affect their ability to present both antigens. B6 LN cells primed with OVA induced antigen-specific responses comparable with those of B6 LN cells primed with Z. Hence, measurement of cell collaboration under in vivo conditions reveals the same requirements for associative recognition as seen in vitro.

F_1 T Cell Populations Primed in a Parental Environment. F_1 T cells were primed to protein antigens in an irradiated parental environment and the genetic requirements for subsequent proliferative and helper activity were determined. The findings (data not shown) with such positively selected cells were identical at every step with the findings described above for negatively selected cells.

Discussion

In the experimental model we have examined, two conclusions are clear: (*a*) T cell-APC interactions require genetic compatibility, whether for proliferation (Figs. 3 and 6) or for helper induction (Figs. 5, 7, and 8); and (*b*) there is no genetic restriction on the B cell receiving the helper activity once help is induced (Figs. 8–10). These findings are compatible with those of Heber-Katz and Wilson (17), Pierce et al. (18), Swain et al. (19), von Boehmer and Sprent (20), Waldmann et al. (21), McDougal

and Cort (22), Singer et al. (23), and those in our forthcoming paper (W. H. Shih, P. Matzinger, S. L. Swain, and R. W. Dutton. Analysis of histocompatibility requirements for proliferative and helper T cell activity: F_1 cells primed in a parental environment. Manuscript in preparation.). They differ from those of Kappler and Marrack (12), Sprent (13, 14), Swierkosz et al. (15), and Yamashita and Shevach (16). We will postpone the discussion of the possible reasons for these conflicting results to later in the discussion.

It is first necessary to discuss the evidence that our findings of collaboration between genetically disparate T and B cells are not a result of allogeneic effects. When unselected carrier primed T cells from the LN are cultured with antigen- and hapten-primed allogeneic B cells there is a strong response. This response is comparable to the one seen with hapten-primed syngeneic cells, but shows no requirement for associated or linked recognition of hapten and carrier determinants (Fig. 1). We conclude that the response is an example of a PAE. The suppression seen at high T cell numbers is taken as a manifestation of an NAE. In the subsequent experiments (Figs. 5, 9, and 10 and Table I) we have determined whether there is a requirement for linked recognition and have taken the findings of a requirement as an indication that a response is not dependent on a PAE. We consider this an important control lacking in most previous studies.

In the experiments presented in this paper we have used a negative-selection procedure of filtration through a irradiated allogeneic host to remove alloreactive T cells. T cell populations taken from the thoracic duct in the period 18–40 h after injection are depleted of cells reactive to the allogeneic host Such populations can then be tested for their interactions with syngeneic or allogeneic cells.

It was demonstrated that the negatively selected cells were markedly depleted of cells reactive to the allogeneic host. Thus there was no proliferative response when the negatively selected cells were cultured with allogeneic APC (Fig. 3 a) and there was no PFC response (PAE) when cultured with allogeneic B cells plus APC (Fig. 4). There was also no evidence of any NAE. Thus the addition of allogeneic APC did not suppress the response of the syngeneic system in the proliferative system (Fig. 3 b). Nor did the addition of allogeneic, but negatively selected, T cells suppress the PFC response of the whole spleen cell population (Fig. 4 b).

The evidence that there is no restriction for T-B interaction rests on the results presented in Fig. 5, 7, and 8. In Fig. 5 BALB/c TDL depleted of cells reactive to B6 will collaborate with B6 B cells (plus APC) but only if BALB/c APC are present. No response was seen when TNP coupled to a heterologous carrier was used as antigen, and by this criterion, the response was not a result of a PAE. In Fig. 8, B10.A T cells depleted of cells reactive with B6 collaborate with B6 B cells purified by two passages through Sephadex G-10 if B10.A APC are present. Once again, the response was not a result of a PAE because the addition of syngeneic APC with TNP on the heterologous carrier had no effect.

There are several further points of interest: (*a*) The addition of allogeneic, antigen-pulsed APC to syngeneic T cells and T-depleted cells (B cells + APC) gave good responses (Fig. 7). This apparent lack of a requirement for syngeneic APC can be explained by the transfer of antigen from allogeneic to syngeneic cells. When syngeneic APC are removed from the T-depleted population by two passages through Sephadex G-10 columns (Fig. 8), the response disappears. It is of interest that a single passage

through Sephadex G-10 was not sufficient. The transfer of very small amounts of antigen on the allogeneic cells to the syngeneic APC seems remarkably effective—perhaps because of cytophilic antibody on the cells of the TNP-primed population. Thus titrations of the response against the number of antigen-pulsed cells show no difference between syngeneic and allogeneic antigen-pulsed cells either in Fig. 7 or in our earlier experiments by Swain and Dutton (31).

(*b*) A striking feature of the results was the fact that APC pulsed separately with the priming carrier and hapten coupled to an unrelated carrier were not functional. This suggests that there are two T cell interactions. The first interaction is between T cell and APC, which is carrier specific and is H-2 restricted. The second is between T cell and B cell, which requires linked recognition, is not H-2 restricted, and does not take place at the surface of the APC. It is possible that these two interactions are sequential events in the functioning of a single T cell population, or it is possible that they represent the interactions of two separate populations of T cells. In the latter case it should be noted that neither T cells is required to interact with the B cell in an H-2-restricted manner.

(*c*) The conditions that govern the T cell-B cell-APC interactions in vitro are also seen in an in vivo model. Thus T cell-APC interactions are genetically restricted, and the T cell-B cell interaction shows a requirement for linked recognition of antigenic determinants, but no requirement for genetic matching.

As noted before, these findings are compatible with those of a number of other investigators (4, 17–19, 21–23). Of these, the most germane to the present discussion are our own earlier studies (19) and those of McDougal and Cort (22) and Singer et al. (23). In our earlier studies alloreactive cells were removed from the primed helper T cell population by treatment with anti-Ly-2 and C. McDougal and Cort (22) used positive selection of F_1 T cells in an in vitro model. Singer et al. (23) carried out an extensive series of experiments with T cells from primed and unprimed F_1 and chimeric mice and demonstrated only a requirement for compatibility between T cells and APC. Our own experiments confirm these observations and extend them in one essential step—namely, the demonstration that, in every case, the collaboration between T cell and incompatible B cell involves linked recognition of the hapten and carrier determinants.

Our results differ from those of Sprent (13, 14), von Boehmer et al. (4), Yamashita and Shevach (16), and Kappler and Marrack and their colleagues (15). In their early studies, von Boehmer et al. (4) showed that histoincompatible T cells and B cells from tetraparental bone marrow chimeras could collaborate in a secondary response to sheep erythrocytes. This was initially interpreted as collaboration across a haplotype barrier which could take place when the two populations were mutually tolerant. Subsequently, however, it was realized that the parental populations injected into the F_1 recipients would be selected in the F_1 thymus to recognize both parental types as self. In later experiments (Sprent and von Boehmer [32]) T cells negatively selected to remove alloreactive cells failed to collaborate with allogeneic B cells in an in vivo secondary response in an irradiated F_1 host. This model is very similar to the one employed by our in vivo experiments and differs only in the antigen, sheep erythrocytes (SRBC) versus hapten-protein conjugates; the immunization schedules employed for the B cells; and the strains of mice employed, B6 and CBA versus B10 and B10.A or B6 and DBA/2 and other seemingly inconsequential details. The difference

in antigens and immunization schedules will be discussed below. Subsequent experiments by Sprent (13, 14) used F_1 T cells that had been simultaneously primed and positively selected in a parental environment. These are comparable to our positive selection experiments. In Sprent's experiments the positively selected cells failed to collaborate with B cells of the opposite parental strain. It was demonstrated that the failure was not a result of suppression or of a lack of APC of the appropriate haplotype. Again, the only significant details in experimental protocol would seem to be in the antigen used and the immunization of the B cells.

In the experiments by Yamashita and Shevach (16) F_1 T cells from primed donors were selected in vitro with antigen on parental APC and then shown to collaborate only with the parental B cells syngeneic to the priming APC. Soluble antigen was added in the second culture in which the collaboration between the selected T cells and the B cells was assayed and the addition of appropriate APC did not overcome the failure to collaborate.

Swierkosz et al. (15) had earlier used a similar protocol to select F_1 T cells by attachment to antigen-pulsed macrophage monolayers. In the study the T cells and B cells were from mice primed to SRBC and the response to SRBC or TNP-SRBC was measured. Again F_1 T cells selected on macrophages of one parental type would not collaborate with B cells of the other parental type. The failure was not a result of suppression or lack of the appropriate APC.

It is clear that these differences are not a result of technical failures. The failures to find collaboration between incompatible T and B cells do not appear to be a result of suppression, and the findings of collaboration are not explained by PAE. We do not have the resolution of this conflict between the opposing sets of results, but a couple of possibilities can be discussed.

One suggestion for reconciling these results has been proposed by Kappler and Marrack and their colleagues (33–35). They suggest that the B cell requires collaboration of two synergizing T cell populations. One T cell (TH_1) is MHC restricted in its interaction with the APC and sends an antigen-specific signal to the responding B cell. This signal is needed to initiate proliferation in response to soluble antigen but is not required in the B cell response to SRBC antigen, in which case the erythrocyte antigen alone can initiate the response. The second T cell (TH_2) is MHC restricted in its interaction with the B cell but sends and can be replaced by a non-antigen-specific signal to the B cell (Marrack et al. [33], Kappler et al. [34], and Keller et al. [35]). They propose that it is the latter cell which is responsible in protocols in which there is a failure to see collaboration between incompatible T and B cells, whereas no restriction is seen in protocols in which a source of the non-specific helper factor is present. It should be noted that other models have been proposed in which there is a requirement for two helper T cells (Tada et al. [36], Woodland and Cantor [37], and Bottomly and Mosier [38]), but there has been no systematic attempt to relate these models one to another.

In our in vitro studies one could imagine that a nonspecific helper factor is generated in the H-2-restricted interaction between T cell and APC replacing the requirement for TH_2. It is difficult, however, to imagine that a sufficient concentration of such a nonspecific factor could be generated in our in vivo experiments described in this paper. Moreover, the requirements that we have defined for the response,

MHC restriction in the T-APC interaction and linked recognition in the T-B interaction are not compatible with the model for TH_1 interactions.

A quite different possibility would be that B cells in different stages of differentiation would have different triggering requirements. In such a model less-differentiated B cells from lightly primed donors would require signals from two types of T cells, whereas more-differentiated B cells from repeatedly immunized donors would need signals from only one of the T cells. In our experiments this T cell would show restriction in its interaction with APC and a requirement for linked recognition in its interaction with the B cell. As discussed, these two interactions could be sequential events in the life of the same T cell or could represent the activities of two separate T cells, neither of which is equivalent to TH_2. The B cells used in our studies are from hyperimmunized donors that had been repeatedly injected with antigen over a protracted period of time and might be expected to contain the hypothetical mature cells. In contrast, Kappler et al. (34) used B cells from donors primed with a single injection of TNP-lipopolysaccharide 7 d before use. In the protocols employed by Sprent (13, 14) the antigen was SRBC and again the B cells were primed by a single exposure to antigen before the demonstration of restriction in T-B collaboration.

It is thus possible that state of B cell differentiation is the determining factor that distinguishes the two sets of conflicting results; however, further studies are required to investigate this point.

Summary

T cell populations were prepared from donors immunized with hapten-carrier conjugates and were depleted of alloreactive cells by negative selection. This was accomplished by injection of the cells into H-2-disparate irradiated recipients and recovery from the thoracic duct after 18–40 h. The genetic requirements for the proliferative and helper activity of these populations was determined. The proliferative response to antigen presented on adherent, Thy-1-negative cells was determined, and a requirement for syngeneic antigen-presenting cells (APC) was demonstrated. The same T cells were assayed for their ability to give help to hapten primed B cells. It was shown that there was a requirement for syngeneic APC and for linked recognition of hapten and carrier determinants on the same molecule by the B cell and T cell. There was no requirement for the B cell to be H-2 compatible with the T cell. The requirement for linked recognition was taken as evidence that the responses in allogeneic combinations were not a result of positive allogeneic effects. Precisely comparable restrictions were found with positively selected cells.

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