HAPLOTYPE-SPECIFIC SUPPRESSION OF ANTIBODY RESPONSES IN VITRO

I. Generation of Genetically Restricted Suppressor T Cells by Neonatal Treatment with Semiallogeneic Spleen Cells

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The development of antibody responses to complex antigens requires interactions among antigen-presenting macrophages (Mφ), B cells, and helper T cell populations that are regulated or restricted by products of the I region (usually the I-A subregion) of the murine H-2 complex (1-8). Although strict genetic restrictions control Mφ-immune T cell interactions in antibody and DNA synthetic responses to antigen (6-9), these restrictions were not apparent in primary plaque-forming cell (PFC) responses to the polymer of l-glutamic acid-l-alanine-l-tyrosine (GAT; 6, 7, 10). Syngeneic or allogeneic Mφ presented GAT with similar efficiency and stimulated comparable PFC responses, suggesting a lack of genetic restrictions in Mφ-virgin T cell interactions. It is, however, virtually impossible to exclude the involvement of allogeneic effects resulting from the accompanying mixed lymphocyte reaction (MLR), even if not detectable by a variety of criteria (6), in responses stimulated by allogeneic Mφ. Therefore, we attempted to induce alloantigen-specific tolerance by injection of semiallogeneic cells into neonates (11-14) to analyze primary responses to GAT stimulated by allogeneic Mφ in the absence of potential complications involving alloreactivity. A problem with this approach is the difficulty of inducing tolerance to antigens encoded by the left half of the H-2 complex (H-2K to I-E region), the regions of interest in mice of the C57BL/10 genetic background, the strain of choice to allow future genetic mapping studies (13, 14).

We failed to induce alloantigen tolerance; MLR and cytotoxic T lymphocyte (CTL) responses were depressed but significant, and skin grafts were rejected normally.

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1 Abbreviations used in this paper: BA, Brucella abortus; C, complement; CTL, cytotoxic T lymphocyte; E:T, effector to target ratio; GAT, random polymer of l-glutamic acid-l-alanine-l-tyrosine; [3H]Tdr, tritiated thymidine; HRBC, horse erythrocytes; KLH, keyhole limpet hemocyanin; Mφ, macrophage; MLR, mixed lymphocyte reaction; PEC, peritoneal exudate cells; PFC, plaque-forming cell; SRBC, sheep erythrocytes; TNP, trinitrophenol; Ts cell, suppressor T cell.

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HAPLOTYPE-SPECIFIC SUPPRESSOR T CELLS

We have, however, demonstrated a population of suppressor T cells (Ts cells) that is activated by I-A antigens of the haplotype encountered neonatally and that nonspecifically suppresses primary PFC responses in culture. This report describes some properties of these Ts cells and the genetic restrictions governing their activation and function, and discusses their possible significance.

Materials and Methods

**Mice.** C57BL/10 (B10), B10.D2, B10.A, BALB/cAn, C3H/HeSn, AKR/J, and (B10.D2 x B10)F1 mice were bred in the animal facility of The Jewish Hospital, St. Louis, Mo, C3H.OL, B10.GD, B10.LG, B10.A(4R), and B10.A(5R) mice were provided by Dr. D. Shreffler (Department of Genetics, Washington University School of Medicine, St. Louis, Mo.); B10.A(3R) mice were provided by Dr. J. Stimppling (McLaughlin Research Institute, Great Falls, Mont.). Mice were maintained on water and laboratory chow ad libitum and used at 6-20 wk of age.

**Antigens.** GAT (~45,000 mol wt; Miles Laboratories Inc., Elkhart, Ind.) was prepared as previously described for use as antigen in culture (15), preparing GAT-Mφ (6), coupling to sheep erythrocytes (SRBC) for use as indicator cells in the hemolytic PFC assay (15), and for in vivo immunization (15). Trinitrophenol (TNP)-derivitized sheep and horse erythrocytes (TNP-SRBC and TNP-HRBC) were prepared by the techniques of Rittenberg and Pratt (16) and used as antigen in culture or as indicator cells in the PFC assay. Keyhole limpet hemocyanin (KLH) and *Brucella abortus* (BA) were derivatized with TNP as described by Zitron et al. (17).

**Immunizations.** Neonatal B10 or BALB/c mice received 20 X 10⁶ syngeneic or (B10.D2 x B10)F1 spleen cells in phosphate-buffered Ringer’s solution, pH 7.6, via the orbital branch of the anterior facial vein within 18 h of birth, as described by Billingham et al. (11, 12). Mice were analyzed for immune reactivity at 6-20 wk of age. Adult mice were immunized by intraperitoneal injections of 10 µg GAT in a mixture of magnesium-aluminum hydroxide (Maalox; W. H. Roher, Inc., Ft. Washington, Pa.) and pertussis vaccine (Eli Lilly, Indianapolis, Ind.; 15) or 4 X 10⁶ of the appropriate GAT-Mφ (bearing ~25 ng GAT/10⁶ cells; 6).

**Preparation of Cell Populations.** T cells were separated from single cell suspensions of lymph node or spleen by passage over nylon wool columns (18). Peritoneal exudate cells (PEC) obtained by flushing the peritoneal cavity of the mice with Hanks’ balanced salt solution containing 10 IU/ml heparin were the source of Mφ. Spleen cells were treated with anti-Thy-1.2 serum and complement (C) and used as T cell-depleted spleen cells (10). Spleen cells were irradiated as indicated using a Gamma-Cell 40 Cs source (Atomic Energy of Canada, Ltd., Ottawa, Canada).

**Culture System and Hemolytic Plaque Assay.** Spleen cells in completely supplemented Eagle’s minimal essential medium with 10% fetal calf serum (lot 32301; Reheis Chemical Co., Kankakee, Ill.) were incubated with 3 X 10⁶ GAT-Mφ, TNP-KLH-Mφ, or soluble antigen as indicated at 5 X 10⁶ cells in 0.6 ml in 16-mm wells of a 24-well tissue culture plate (FB-16-24-TC; Flow Laboratories, Inc., Linbro Chemical Co., Hameden, Conn.) for 5 d under modified Mishell-Dutton conditions (19). GAT-Mφ were prepared using appropriate PEC as previously described (6) and had ~2-4 ng GAT/10⁶ cells. TNP-KLH-Mφ were prepared by incubating 2 X 10⁶ PEC with 100 µg/ml TNP-KLH in serum-free medium at 37°C for 60 min. Cells were washed, resuspended in medium, and added to culture. IgG GAT-specific, IgM and IgG anti-SRBC, or IgM and IgG TNP-specific PFC responses were assayed using the slide modification of the Jerne hemolytic plaque assay (19). Data are expressed as PFC/culture. In all experiments, neonatally treated mice were examined individually.

**MLR.** Spleen or lymph node cells (2.5 X 10⁵) from responder mice were incubated with an equal number of mitomycin C-treated (50 µg/ml; Sigma Chemical Co., St. Louis, Mo.) or irradiated (2,000 rad) stimulator spleen cells from mice of appropriate H-2 haplotypes in RPMI-1640 supplemented with 2 mM L-glutamine, antibiotics, 5 X 10⁻⁵ M 2-mercaptoethanol, and 5% fetal calf serum (Reheis Chemical Co.) in 96-well round-bottomed microtiter plates (Dynatech Laboratories Inc., Dynatech Corp., Alexandria, Va.), in 0.2 ml in 5% CO₂ in air. After 96 h, each well received 1 µCi of tritiated thymidine ([³H]TdR; 6.7 Ci/mM sp ac; Research Products International Corp., Elk Grove Village, Ill.) and was harvested 18 h later using a Titerette Cell Harvester (Flow Laboratories, Inc., Rockville, Md.). Incorporation of...
[^H]TdR into DNA was determined in a Beckman Scintillation Counter (model LS-333; Beckman Instruments Inc., Fullerton, Calif.). Data are presented as mean cpm incorporation in triplicate wells.

**CTL Responses.** Responder spleen cells (7 × 10^6 cells) were incubated with irradiated (2,000 rad) stimulator spleen cells (3 × 10^6 cells) in 2 ml of supplemented RPMI-1640 containing 10% fetal calf serum in 16-mm wells of a 24-well Linbro plate in 5% CO₂ in air for 5 d. At assay, cells were adjusted to appropriate viable cell concentrations and added to the appropriate ^51Cr-labeled tumor target cells at the indicated effector to target (E:T) cell ratios. These mixtures were incubated in 96-well round-bottomed microtiter plates (Dynatech Laboratories, Inc.) for 4 h at 37°C; supernates were harvested and the amount of ^51Cr released was determined in a gamma spectrometer (Beckman Instruments, Inc.). Percent specific ^51Cr release was calculated as follows:

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\text{percent release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.
\]

Total release was counts released by 1% sodium dodecyl sulfate.

**Results**

**Immune Responses by Spleen Cells from Neonatally Treated Mice.** Spleen cells from adult B10 (H-2^b^) mice injected as neonates with 20 × 10^6 syngeneic B10 spleen cells developed significant MLR and CTL responses to allogeneic AKR (H-2^k^) and B10.D2 (H-2^d^) stimulator cells (Table I, Fig. 1). Spleen cells from adult mice injected as neonates with 20 × 10^6 semiallogeneic [B10.D2 × B10 (H-2^d^ × b)]F1 spleen cells developed normal MLR and CTL responses to allogeneic AKR cells and lower, but nevertheless significant, responses to B10.D2 stimulator cells, the allogeneic haplotype encountered neonatally (Table II, Fig. 1). This reactivity to H-2^d^ stimulator cells suggested that at best only partial tolerance had been induced. Complete unreactivity to H-2^d^ stimulator cells in MLR and CTL responses could not be demonstrated under any conditions where the numbers of stimulator or responder cells or the time of assay were varied. Moreover, B10 mice treated as neonates with (B10.D2 × B10)F1 spleen cells rejected first-set H-2^d^ skin grafts with the same kinetics, 16–17 d, as normal B10 mice or B10 mice treated with syngeneic spleen cells as neonates. These mice also failed to develop responses to H-2^d^ antigens in vivo, but developed significant titers to H-2^k^ antigens. An identical pattern of reactivity was observed in BALB/c (H-2^d^) mice treated as neonates with (B10.D2 × B10)F1 spleen cells; these spleen cells had

| Stimulator cells |[^H]TdR incorporation (cpm ± SEM) | responder cells |
|------------------|---------------------------------|----------------|
| B10              | 4,400 (1.02)                    | 4,100 (1.05)   |
| AKR              | 11,500 (1.11)                   | 11,880 (1.06)  |
| B10.D2           | 14,300 (1.04)                   | 8,200 (1.03)   |

Spleen cells from adult B10 mice injected as neonates with B10 spleen cells (B10c) or (B10.D2 × B10)F1 spleen cells (B10t) were cultured at 2.5 × 10^5 cells with an equal number of the indicated irradiated (2,000 rad) stimulator spleen cells. The cultures received 1 µCi of[^H]TdR on day 3, were harvested 18 h later, and assayed for incorporation of[^H]TdR. Data are expressed as geometric mean cpm of triplicate cultures; ± SEM are in parentheses.
reduced, but significant, reactivity against H-2^b^ stimulator cells in MLR and CTL responses and the mice rejected H-2^b^ skin grafts normally. Finally, adult B10 or BALB/c mice injected intravenously with 20 × 10^6^ (B10.D2 × B10)F1 spleen cells had enhanced MLR and CTL responses when stimulated 4 wk later with cells of the allogeneic H-2^d^ haplotype used for injection (data not shown).

Spleen cells from B10 mice treated as neonates with (B10.D2 × B10)F1 spleen cells were also tested for their ability to develop primary PFC responses in vitro when stimulated with GAT presented on Mφ of various H-2 haplotypes (Table II). Spleen cells from normal or control B10 mice developed significant responses to soluble GAT and to GAT-Mφ from syngeneic B10 and allogeneic AKR and B10.D2 mice. Spleen cells from neonatally treated B10 mice responded to soluble GAT and to GAT presented on syngeneic B10 and unrelated allogeneic AKR-Mφ, but failed to respond to B10.D2 or (B10.D2 × B10)F1 GAT-Mφ. Neonatally treated mice also failed to respond to SRBC in the presence of B10.D2 Mφ but responded at control levels in the

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**Table II**

Primary In Vitro PFC Responses by Spleen Cells from Neonatally Treated Mice

| Antigens and Mφ added | Day 5 PFC/culture* |
|-----------------------|--------------------|
|                       | B10,‡               | B10,‡               |
| GAT                   | 870                | 680                |
| B10 GAT-Mφ            | 905                | 1,083              |
| AKR GAT-Mφ            | 675                | 618                |
| B10.D2 GAT-Mφ         | 663                | <10                |
| (B10.D2 × B10)F1 GAT-Mφ| 698               | <10                |
| SRBC                  | 2,465              | 3,830              |
| SRBC + AKR-Mφ         | 3,380              | 3,300              |
| SRBC + B10.D2 Mφ      | 3,980              | 370                |

* Spleen cells (5 × 10^6^) were cultured under Mishell-Dutton conditions with 2 μg soluble GAT, 3 × 10^6^ GAT-Mφ from the indicated strain bearing 3-4 ng GAT 10^6^ cells, 10^7^ SRBC, or SRBC plus 3 × 10^6^ Mφ from the indicated strain. IgG GAT-specific PFC/culture; IgM PFC/culture vs. SRBC.

‡ B10 indicates B10 mice injected with B10 spleen cells as neonates; B10M indicates B10 mice injected with (B10.D2 × B10)F1 spleen cells as neonates.
presence of allogeneic AKR Mφ. An identical pattern of responsiveness was observed in BALB/c spleen cells from mice treated as neonates with (B10.D2 × B10)F₁ spleen cells; these spleen cells failed to respond to B10 GAT-Mφ but responded to BALB/c and AKR GAT-Mφ and soluble GAT (data not shown). In all experiments, responses to SRBC in the absence of added syngeneic or allogeneic Mφ were comparable.

Demonstration of Haplotype-specific Ts Cells. The role of active suppression as the basis for the specific failure to respond to GAT-Mφ of the H-2 haplotype that the mice were exposed to as neonates was investigated. Spleen cells or T cells from control or neonatally treated B10 mice were added to normal B10 spleen cells in cultures stimulated with GAT-Mφ of various H-2 haplotypes (Table III). Control B10 spleen cells (experiment 1) or T cells (experiment 2) did not alter the responses of normal B10 spleen cells to B10, AKR, or B10.D2 GAT-Mφ. Spleen cells or T cells from B10 mice injected with (B10.D2 × B10)F₁ spleen cells as neonates did not alter the responses of normal B10 spleen cells to B10 or AKR GAT-Mφ; however, responses to B10.D2 GAT-Mφ were completely suppressed. This degree of suppression was routinely observed with 10⁸ whole spleen cells or 0.5 × 10⁶ T cells from spleen or lymph node; however, thymocytes (up to 2 × 10⁶/culture) from treated mice failed to

### Table III

| Experiment | GAT-Mφ     | Day 5 IgG GAT-specific PFC/culture* |
|------------|------------|-----------------------------------|
|            |            | Normal spleen cells | Treated spleen cells |
| 1          | B10        | 708 | 615 |
|            | AKR        | 510 | 600 |
|            | B10.D2     | 620 | <10 |
| 2          | B10        | 295 | 295 |
|            | AKR        | 360 | 310 |
|            | B10.D2     | 290 | <10 |
| 3          | B10        | 550 | 655 |
|            | AKR        | 588 | 745 |
|            | B10.D2     | <10 | 718 |

* B10 spleen cells, 5 × 10⁶, were cultured under Mishell-Dutton conditions with 3 × 10⁴ GAT-Mφ from the indicated strain bearing 3–4 ng GAT 10⁵ cells. Responses of B10 spleen cells stimulated with the various GAT-Mφ without added cells were comparable to the responses in the presence of normal spleen cells and normal T cells.

† Spleen cells (10⁶/culture); nylon wool column-passed spleen cells (T cells; 0.5 × 10⁶/culture). Treated cells from B10 mice injected with (B10.D2 × B10)F₁ spleen cells as neonates; normal cells were nontreated B10 cells.

§ Spleen cells from neonatally treated mice were untreated (control) or treated with anti-Thy-1.2 plus C or with 350 rad before addition to culture.
suppress (data not shown). Analogous results have been obtained from BALB/c mice treated as neonates with (B10.D2 × B10)F1 spleen cells when B10 GAT-Mφ were present in the assay cultures. Finally, the appropriate allogeneic Mφ or the appropriate alloantigens on F1 Mφ added to culture were sufficient to activate the suppressor cell (data not shown). Comparable viable cell recovery in all cultures suggested that the suppression was not the result of a selective cytotoxic mechanism.

The activity of these suppressor cells was completely abrogated by treatment with anti-Thy-1.2 plus C and as little as 350 rad gamma irradiation (Table III). More complete characterization of these Ts cells will be the subject of a future communication. The addition of normal T cells to spleen cells from neonatally treated mice, depleted of T cells, restored the capacity to develop PFC responses to allogeneic GAT-Mφ of the haplotype encountered as neonates. These results suggested that the B cell pool was intact and unaffected by the neonatal treatment that had induced a population of radiosensitive, haplotype-specific Ts cells.

Parameters of Suppression by Haplotype-specific Ts Cells. These haplotype-specific Ts cells also inhibited primary responses to SRBC, and anti-TNP PFC responses by normal syngeneic spleen cells to TNP-SRBC, TNP-KLH, TNP-FicolI, and TNP-BA. IgM PFC responses were suppressed, but to a lesser extent than IgG responses, and responses to particulate antigens (TNP-SRBC, SRBC, and TNP-BA) were more resistant to suppression than responses to soluble antigens (data not shown). These results demonstrated that these Ts cells inhibit IgM and IgG PFC responses to soluble and particulate, and T cell-dependent and T cell-dependent antigens (TNP-BA and TNP-FicolI).

The kinetics of suppression of primary PFC responses by haplotype-specific Ts cells was investigated under conditions in which the Ts cells were activated by adding the relevant Mφ at various times after the initiation of cultures containing normal B10 spleen cells and B10 spleen cells from neonatally treated mice (Table IV). Complete suppression of day 5 responses was observed only when the allogeneic B10.D2 Mφ were added within 24 h of culture initiation. Suppression was significant but incomplete when the Ts cells were activated at 36 h and was more variable, ranging from 0 to ~50%, when Ts cells were activated at 48 h. Higher numbers of Mφ added at or after 48 h were unable to activate Ts cells to suppress day 5 responses (data not shown). Ts cells, preactivated with the appropriate Mφ for 48 h, failed to suppress PFC responses completely when added to culture 48 h after initiation, but when added at culture initiation or 24 h after culture initiation, the Ts cells blocked initiation of the PFC responses.

The ability of these haplotype-specific Ts cells to suppress secondary PFC responses to GAT was examined. Spleen cells from mice primed with syngeneic or allogeneic GAT-Mφ developed secondary responses to GAT only when stimulated with GAT-Mφ syngeneic to the Mφ used for priming in vivo (6, 20). B10 mice were immunized with B10, B10.D2, or (B10.D2 × B10)F1 GAT-Mφ; spleen cells from these mice developed secondary PFC responses only to the GAT-Mφ syngeneic to those used for immunization (Table V). Ts cells from neonatally treated B10 mice, which suppressed primary PFC responses when exposed to the appropriate allogeneic GAT-Mφ, failed to suppress secondary PFC responses to the same GAT-Mφ. Thus, although these Ts cells can suppress PFC responses to a variety of antigens, only primary responses were
Table IV

Kinetics of Suppression by Haplotype-specific Ts Cells

| Time of addition of B10.D2 Mφ | Day 5 IgG GAT-specific PFC/culture |
|------------------------------|-----------------------------------|
|                              | Normal cells                      |
|                              | Treated cells                     |
| Experiment 1                 |                                   |
| 0 h                          | 433                               |
| 24 h                         | 438                               |
| 36 h                         | 405                               |
| 48 h                         | 395                               |
| Experiment 2                 |                                   |
| Time of addition of activated Ts cells | Normal cells |
|                              | Treated cells                     |
| 0 h                          | 413                               |
| 24 h                         | 463                               |
| 48 h                         | 400                               |

In experiment 1, 5 × 10^6 spleen cells were cultured with 10^6 spleen cells from normal mice or B10 mice injected neonatally with (B10.D2 × B10)F1 spleen cells and 2 μg GAT. At the indicated times, 3 × 10^4 B10.D2 PEC were added. In experiment 2, spleen cells from normal or neonatally treated B10 mice were incubated with 3 × 10^6 B10.D2 PEC for 48 h and added as indicated to cultures of 5 × 10^6 normal B10 mice and 2 μg GAT.

Table V

Haplotype-specific Ts Cells Are Unable to Suppress Secondary Responses to GAT

| GAT-Mφ used for priming* | T cells§ | Day 5 IgG GAT-specific PFC/culture§ |
|---------------------------|----------|------------------------------------|
|                           | Normal   | B10                                |
|                           | Treated  | B10.D2                             |
| None                      | Normal   | GAT-Mφ                             |
|                           | Treated  | GAT-Mφ                             |
| B10                       | Normal   | 840                                |
|                           | Treated  | 715                                |
| B10.D2                    | Normal   | 740                                |
|                           | Treated  | 733                                |
| (B10.D2 × B10)F1          | Normal   | <10                                |
|                           | Treated  | 840                                |
|                           | Treated  | 778                                |

* B10 mice were primed in vivo 4 wk before assay by intraperitoneal injection of 4 × 10^6 GAT-Mφ from the indicated strain bearing ~25 ng GAT/10^6 cells.
§ Nylon wool column-passed T cells from normal B10 mice or B10 mice injected with (B10.D2 × B10)F1 spleen cells as neonates.
§§ B10 spleen cells, 5 × 10^6, cultured with 0.5 × 10^6 normal or treated B10 T cells and 3 × 10^6 of the indicated GAT-Mφ bearing 3-4 ng GAT/10^6 cells.

suppressed. Moreover, neonatally treated mice cannot be primed using GAT-Mφ against which the Ts cells are directed.

Finally, these haplotype-specific Ts cells were unable to suppress either CTL or MLR to the B10.D2 alloantigens (Table VI), although the same Ts cells suppressed
TABLE VI

Haplotyp-specific Ts Cells Are Unable to Suppress CTL and MLR Responses of Normal Spleen Cells

| Responder* | T cells* | CTL response assay, specific \(^{51}\text{Cr}\) release\(\dagger\) | MLR assay, \(^{3}\text{H}\)-TdT incorporation\(\dagger\) |
|-----------|---------|---------------------------------|---------------------------------|
| B10c      | None    | 20:1 90                         | 31,535 (1.05)                   |
| 5:1       | 74      |
| B10c      | 1.5 \times 10^6 control | 20:1 90                         | 34,897 (1.11)                   |
| 5:1       | 80      |
| B10c      | 1.5 \times 10^6 treated  | 20:1 80                         | 34,300 (1.08)                   |
| 5:1       | 63      |
| B10c      | 0.5 \times 10^6 treated  | 20:1 98                         | 33,594 (1.06)                   |
| 5:1       | 75      |
| B10c      | None    | 20:1 40                         | 12,213 (1.05)                   |
| 5:1       | 15      |

* B10c, or control: B10 mice injected with B10 spleen cells as neonates. B10t, or treated: spleen cells from mice injected with (B10.D2 \times B10)F\(_1\) spleen cells as neonates.
\(\dagger\) 7 \times 10^6 normal (B10c) or treated (B10t) spleen cells were cultured with 3 \times 10^6 irradiated (2,000 rad) B10.D2 stimulator spleen cells for 5 d with the indicated number of T cells from normal or treated mice; percent specific \(^{51}\text{Cr}\) release at the indicated E:T cell ratios on P815 target cells was determined. Spontaneous release: P815 (9%).
\(\dagger\) 200-\mu l aliquot removed at 72 h and pulsed with 1 \mu Ci \(^{3}\text{H}\)-TdT for 18 h. Data are presented as cpm \(\pm\) SEM.

Thus, these Ts cells were unable to suppress proliferation or cytotoxic lymphocyte responses by T cells to alloantigens for which they were specific.

Genetic Constraints Governing Activity of Haplotyp-specific Ts Cells. The subregion(s) of the H-2 complex responsible for the activation and restriction of activity of these Ts cells was determined using congenic and recombinant strains of mice in mapping studies. First, using Mφ from mice that shared regions of the H-2 complex with the allogeneic parent of the F\(_1\) cells administered to the neonates, the genetic requirements for activation of the Ts cells were determined (Table VII). B10.D2, C3H.OL, and B10.GD, but not B10.LG, GAT-M\(_1\) activated B10 Ts cells specific for the H-2\(^d\) haplotype resulting in suppression of responses by normal B10 spleen cells. Thus, syngenicity at the I-A subregion of the H-2 complex was necessary and sufficient for activation of Ts cells. Second, the genetic restrictions on haplotyp-specific Ts cell activity were determined using Ts cells activated by the appropriate GAT-Mφ and normal responder spleen cells that shared various regions of the H-2 complex (Table VIII). This analysis was potentially complicated because Ts cells and responding cells differ at some regions of the H-2 complex. In experiment 1, responses by B10, B10.A(3R), and B10.A(5R) spleen cells were suppressed by B10 Ts cells activated by the B10.D2 Mφ, placing the restriction in the K, I-A, and I-B subregions and definitely eliminating syngenicity at the I-J subregion as a requirement for expression of suppressive activity. The data in experiment 2 demonstrated that syngenicity at the I-A subregion of H-2 was necessary and sufficient for the Ts cells to express activity.
Table VII

**Genetic Requirements for Activation of Haplotype-specific Ts Cells**

| GAT-Mφ*         | Shared H-2 regions† | Control T cells‡ | Treated T cells§ |
|-----------------|---------------------|------------------|------------------|
| B10.D2          | KABJECGD            | 535              | 10               |
| B10.A           | CSGD                | 563              | 575              |
| C3H.OL          | KABJEC              | 508              | <10              |
| B10.GD          | KA                  | 573              | <10              |
| B10.LG          | K                   | 500              | 488              |
| C3H             | None                | 588              | 585              |

* 3 × 10⁴ GAT-Mφ/culture bearing ~3-4 ng GAT/10⁵ cells.
† H-2 regions shared between the stimulating GAT-Mφ and the B10.D2 haplotype for which the Ts cells are specific.
‡ B10 spleen cells, 5 × 10⁶, were cultured with the indicated GAT-Mφ and 10⁶ control or neonatally treated B10 T cells under Mishell-Dutton conditions.
§ Control: B10 mice injected with B10 spleen cells as neonates; treated: B10 mice injected with (B10.D2 × B10)F₁ spleen cells as neonates.

Table VIII

**Genetic Restrictions on Haplotype-specific Ts Cell Activity**

| Experiment | Responder spleen cells | GAT-Mφ* | Shared H-2 regions‡ | Control T cells§ | Treated T cells§ |
|------------|------------------------|---------|---------------------|------------------|------------------|
| 1          | B10                    | B10.D2  | KABJECGD            | 448              | <10              |
|            | B10.A(4R)              | B10.D2  | BJECGD              | 348              | 390              |
|            | B10.A(3R)              | B10.D2  | KAB                 | 440              | <10              |
|            | B10.A(5R)              | B10.D2  | KAB                 | 335              | <10              |
| 2          | B10.D2                 | B10     | KABJECGD            | 485              | <10              |
|            | B10.GD                 | B10     | KA                  | 470              | <10              |
|            | B10.LG                 | B10     | K                   | 503              | 545              |

* 3 × 10⁴ GAT-Mφ/culture bearing ~3-4 ng/10⁵ cells.
† H-2 regions shared between the T cells and the responder spleen cells.
‡ Responder spleen cells, 5 × 10⁶ of the indicated strains were cultured with GAT-Mφ and 10⁶ control or treated T cells under Mishell-Dutton conditions.
§ Experiment 1: control B10 mice were injected with B10 spleen cells as neonates; treated B10 mice were injected with (B10.D2 × B10)F₁ spleen cells as neonates. Experiment 2: control B10.D2 mice were injected with B10.D2 spleen cells as neonates; treated B10.D2 mice were injected with (B10.D2 × B10)F₁ spleen cells as neonates.

Discussion

Mice injected within 18 h of birth when semiallogeneic F₁ spleen cells, using a protocol for induction of alloantigen-specific tolerance described by Billingham et al. (11, 12), were not unresponsive to antigens of the allogeneic haplotype in the F₁ donor. These mice rejected skin grafts of the allogeneic haplotype with normal kinetics; spleen cells from these treated mice developed reduced but significant MLR and CTL responses when stimulated with the appropriate alloantigens. Spleen cells from these mice, however, did not develop primary in vitro PFC responses when Mφ from the allogeneic haplotype encountered as neonates were present in the culture. This
unresponsiveness was due to a population of radiosensitive haplotype-specific Ts cells that were activated by the alloantigens encountered as neonates. These Ts cells nonspecifically suppressed in vitro primary, but not secondary, PFC responses to a variety of antigens and failed to suppress MLR and CTL responses. These Ts cells were activated by cells that shared antigens encoded by the I-A subregion of the H-2 complex with the allogeneic parent in the F1 cells encountered neonatally. Moreover, syngenicity at the I-A subregion between the Ts cell and its target responding spleen cells was necessary and sufficient to mediate suppression.

Active T cell-mediated suppression has been demonstrated to regulate immune responses in a variety of experimental systems. One example analogous to the findings reported here is the MLR suppressor system described by Rich and Rich (21, 22), in which a genetically restricted population of Ts cells capable of inhibiting a primary MLR was demonstrated. These Ts cells were stimulated by immunizing parental strains of mice with appropriate F1 spleen cells; 4 d later, Ts cells were tested for activity in a primary MLR. We were unable to demonstrate suppression of the MLR under any of the conditions of our assays, unlike the suppression evident in the MLR suppressor system, although the mechanism of stimulation of the two types of Ts cells were similar. Another important difference in the two systems is the genetic constraints on the Ts cells. The MLR Ts cell required syngenicity at the I-E/C subregion of the H-2 complex with its target cell (23), whereas the haplotype-specific Ts cell described here required syngenicity at the I-A subregion, both at the level of activation and at the effector stage. Further, the MLR Ts cell had a relatively short life span; activity was maximal 4 d after induction but was not demonstrable by 7 d. In contrast, although the presence of haplotype-specific Ts cells has not been investigated at less than 8 wk after injection of F1 spleen cells, Ts cells have been demonstrated as late as 5 mo after treatment, suggesting that these Ts cells were a relatively long-lived population.

These two Ts cell systems, although independent phenomena, shared some characteristics; both are examples of Ts cells not restricted by the I-J subregion of the H-2 complex, in contrast to antigen specific Ts cells (24, 25). These Ts cells, however, are stimulated and interact with target cells in a genetically restricted manner controlled by the I-A subregions. It should be noted that the absence of I-J determinants on the Ts cell has not been demonstrated, only that syngenicity at the I-J subregion is irrelevant with respect to function. These two cell systems also demonstrate a phenomenon observed with some antigen-specific Ts cells, a certain degree of nonspecificity at the effector stage after activation (26, 27). The haplotype-specific Ts cell after activation by the appropriate stimulus inhibits the primary antibody responses by any spleen cells syngeneic at the I-A subregion, whereas the MLR Ts cell inhibits responses by any responder cell syngeneic at the I-E/C subregions (23).

Important considerations concerning the possible mechanism(s) of action of these Ts cells are the failure to inhibit T cell responses and secondary antibody responses, and required presence of Ts cells during the first 36 h of culture to achieve significant suppression of the primary response; similar constraints have previously been reported for the GAT system (28). The kinetics of suppression suggest that the regulatory step occurs early in the generation of the primary antibody response; thus appropriate target cells may not be required or susceptible in responses by primed spleen cells or virgin spleen cells 48 h or more after culture initiation. This implies that the target
cell(s), possibly an amplifier T cell, is necessary for the primary, but not the secondary, response and is not the same amplifier T cell involved in the MLR; the precedent for such a difference has been previously suggested (29). Alternatively, the unprimed B cell may be the target cell; further studies to determine the target cells of these Ts cells are in progress.

It is important to consider some of the problems inherent in the tolerance system used in this study that may bear on the findings. The inability to induce tolerance by classical criteria is potentially disturbing, but appears to be a function of both the B10 genetic background of the mice used and the relative difficulty of inducing tolerance to antigens of the left half of the H-2 complex (13, 15, 30, 31); higher doses of F1 cells administered to the neonates also failed to induce tolerance. Attempts to induce tolerance in neonatal BALB/c mice using (BALB/c × A)F1 (CAF1) cells, thus eliminating the problem of the B10 background and reducing the antigenic differences at H-2, have resulted in a higher incidence of tolerance by MLR and CTL responses and skin graft rejection but, interestingly, the phenomenon of haplotype-specific Ts cells as reported here were unchanged (C. Sorensen and C. W. Pierce, unpublished data). More difficult observations to explain are the “partial tolerance” induced to the appropriate alloantigens in MLR and CTL responses, but the complete unresponsiveness observed in antibody responses in the presence of the appropriate alloantigens. This is compounded by the observations that the Ts cells responsible for lack of antibody responses are unable to suppress MLR and CTL responses. This paradox is under investigation.

A final point concerns the potential mechanism of action of these Ts cells. Most Ts cell systems described to date involve a soluble mediator; this system is not an exception. The demonstration and characterization of a soluble factor mediating haplotype-specific suppression is the subject of the accompanying report (32).

Summary

C57BL/10 mice were injected with semiallogeneic (B10.D2 × C57BL/10)F1 spleen cells via the anterior facial vein within 24 h of birth to induce tolerance to B10.D2 (H-2d) alloantigens. Spleen cells from these mice as adults developed reduced, but significant, mixed lymphocyte and cytotoxic lymphocyte responses in vitro to H-2d stimulator cells and these treated mice rejected first-set B10.D2 skin grafts within a normal time-course, indicating that at best only a state of partial tolerance had been induced. Spleen cells from these mice failed to develop antibody responses to a variety of antigens in vitro when H-2d macrophages were in the cultures. Partially purified T cells from these neonatally treated mice suppressed primary antibody responses by normal syngeneic spleen cells in the presence of H-2d but not other allogeneic macrophages. These radiosensitive, haplotype-specific suppressor T (Ts) cells inhibited primary antibody responses by blocking initiation of the response, but failed to suppress secondary antibody responses and mixed lymphocyte or cytotoxic lymphocyte responses by appropriate responding spleen cells. To activate H-2d haplotype-specific Ts cells, stimulation with I-Ad subregion antigen(s) was necessary and sufficient; syngenicity at the I-A subregion of H-2 between the activated Ts cells and target responding spleen cell populations was also necessary and sufficient to achieve suppression. Comparable results have been obtained with spleen cells from BALB/c mice injected as neonates with (B10.D2 × C57BL/10)F1 spleen cells where I-Ab
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antigens activate the haplotype-specific Ts cells. Implications for the significance of this population of haplotype-specific Ts cells in immune regulation are discussed and the properties of these Ts cells are compared and contrasted with other antigen-specific and nonspecific Ts cells whose activity is restricted by I-region products.

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References
1. Katz, D. H. 1977. Lymphocyte Differentiation, Recognition and Regulation. Academic Press, Inc., New York. 749.
2. Katz, D. H., M. Graves, M. E. Dorf, H. Dimuzio, and B. Benacerraf. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the I region of the H-2 complex. J. Exp. Med. 141: 263.
3. Pierce, S. K., and N. R. Klinman. 1975. The allogeneic bisection of carrier-specific enhancement of monoclonal B-cell responses. J. Exp. Med. 142: 1165.
4. Sprent, J., and H. von Boehmer. 1976. Helper function of T cells depleted of alloantigen-reactive lymphocytes by filtration through irradiated F1 hybrid recipients. I. Failure to collaborate with allogeneic B cells in a secondary response to sheep erythrocytes measured in vitro. J. Exp. Med. 144: 617.
5. Erb, P., and M. Feldmann. 1975. The role of macrophages in the generation of T-helper cells. II. The genetic control of the macrophage-T-cell interaction for helper cell induction with soluble antigens. J. Exp. Med. 142: 460.
6. Pierce, C. W., J. A. Kapp, and B. Benacerraf. 1976. Regulation by the H-2 gene complex of macrophage-lymphoid cell interactions in secondary antibody responses in vitro. J. Exp. Med. 144: 371.
7. Pierce, C. W. 1980. Macrophages: modulators of immunity. Am. J. Pathol. 98: 9.
8. Rosenthal, A. S., and E. M. Shevach. 1976. The function of macrophages in T-lymphocyte antigen recognition. Contemp. Top. Immunobiol. 5: 47.
9. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interactions between antigen-presenting cells and primed T lymphocytes: an assessment of Ir gene expression in the antigen-presenting cell. Immunol. Rev. 40: 153.
10. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1973. Genetic control of immune responses in vitro. II. Cellular requirements for the development of primary plaque-forming cell responses to the random terpolymer l-glutamic acid30-l-alanine30-l-tyrosine10 (GAT) by mouse spleen cells in vitro. J. Exp. Med. 138: 1121.
11. Billingham, R. E., L. Brent, and P. B. Medawar. 1953. 'Actively acquired tolerance' of foreign cells. Nature (Lond.). 172: 603.
12. Billingham, R. E., and L. Brent. 1956. Acquired tolerance of foreign cells in newborn animals. Proc. R. Soc. Lond. B Biol. Sci. 146: 78.
13. Streilein, J. W., and J. Klein. 1977. Neonatal tolerance induction across regions of H-2 complex. J. Immunol. 119: 2147.
14. Streilein, J., and J. Klein. 1980. Neonatal tolerance of H-2 alloantigens. I. I-region modulation of tolerogenic potential of K and D antigens. Proc. R. Soc. Lond. B Biol. Sci. 207: 461.
15. Kapp, J. A., C. W. Pierce, and B. Benacerraf. 1973. Genetic control of immune responses in vitro. I. Development of primary and secondary plaque forming cell responses to the
random terpolymer L-glutamic acid°°-L-alanine°°-L-tyrosine°° (GAT) by mouse spleen cells
in vitro. J. Exp. Med. 138:1107.

16. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenol (TNP) plaque assay. Primary
response of BALB/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med.
132:575.

17. Zitron, I., D. Mosier, and W. Paul. The role of surface IgD in the response to thymic
independent antigens. J. Exp. Med. 146:1707.

18. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation
of functional thymus derived lymphocytes. Eur. J. Immunol. 3:645.

19. Pierce, C. W., B. M. Johnson, H. E. Gershon, and R. Asofsky. 1971. Immune responses in
vitro. III. Development of primary γM, γG and γA plaque-forming cell responses in mouse
spleen cell cultures stimulated with heterologous erythrocytes. J. Exp. Med. 134:395.

20. Pierce, C. W., and J. A. Kapp. 1978. Suppressor T-cell activity in responder × nonresponder
(C57BL/10 × DBA/1)F1 spleen cells responsive to L-glutamic acid°°-L-alanine°°-L-tyro-
sine°°. J. Exp. Med. 148:2382.

21. Rich, S., and R. Rich. 1974. Regulatory mechanisms in cell-mediated immune responses.
I. Regulation of mixed lymphocyte reactions by alloantigen-activated thymus-derived
lymphocytes. J. Exp. Med. 140:1588.

22. Rich, S., and R. Rich. 1975. Regulatory mechanisms in cell-mediated immune responses.
II. A genetically restricted suppressor of mixed lymphocyte reactions released by alloanti-
gen-activated spleen cells. J. Exp. Med. 142:1391.

23. Rich, S., and R. Rich. 1976. Regulatory mechanisms in cell mediated immune responses.
III. I-region control of suppressor cell interactions with responder cells in mixed lymphocyte
reactions. J. Exp. Med. 143:572.

24. Okumura, K., L. A. Herzenberg, D. B. Murphy, H. O. McDevitt, and L. A. Herzenberg.
1976. Selective expression of H-2 (I-region) loci controlling determinants on helper and
suppressor T lymphocytes. J. Exp. Med. 144:685.

25. Tada, T., M. Taniguchi, and C. S. David. 1976. Properties of the antigen-specific
suppressive T-cell factor in the regulation of antibody responses in the mouse. IV. Special
subregion of the gene(s) that codes for the suppressive T-cell factor in the H-2 histocom-
patibility complex. J. Exp. Med. 144:713.

26. Taylor, R. B., and A. Basten. 1976. Suppressor cells in humoral immunity and tolerance.
Br. Med. Bull. 32:152.

27. Elson, C. J., and R. B. Taylor. 1974. The suppressive effect of carrier priming on the
response to a hapten-carrier conjugate. Eur. J. Immunol. 4:682.

28. Kapp, J. A., C. W. Pierce, S. Schlossman, and B. Benacerraf. 1974. Generic control of
immune responses in vitro. V. Stimulation of suppressor T cells in nonresponder mice by the
terpolymer L-glutamic acid°°-L-alanine°°-L-tyrosine°° (GAT). J. Exp. Med. 140:468.

29. Cantor, H., J. Hugenerberger, L. McVay-Boudreau, D. D. Eardley, J. Kemp, F. W. Shen,
and R. K. Gershon. 1978. Immunoregulatory circuits among T-cell sets. Identification of
a subpopulation of T-helper cells that induces feedback inhibition. J. Exp. Med. 148:871.

30. Kuperman, O. J., H. W. Sollinger, and F. H. Bach. 1977. Tolerance induction to H-2
central region target antigens: in vitro/in vitro correlations. Scand. J. Immunol. 6:553.

31. Holan, V., J. Chutna, and M. Hasek. 1978. Participation of H-2 regions in neonatally
induced transplantation tolerance. Immunogenetics. 6:396.

32. Sorensen, C. M., and C. W. Pierce. 1981. Haplotype-specific suppression of antibody
responses in vitro. II. Suppressor factor produced by T cells and T cell hybridomas from
mice treated as neonates with semiallogeneic spleen cells. J. Exp. Med. 153:48.