LONG-LASTING SKIN ALLOGRAFT TOLERANCE IN ADULT MICE INDUCED ACROSS FULLY ALLOGENEIC (MULTIMAJOR H-2 PLUS MULTIMINOR HISTOCOMPATIBILITY) ANTIGEN BARRIERS BY A TOLERANCE-INDUCING METHOD USING CYCLOPHOSPHAMIDE

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One goal of transplantation immunology is to make possible successful tissue and organ transplantation without need for long-term immunosuppression (1). To overcome immunological barriers that include the strongest multimajor histocompatibility (H)1 complex antigenic barriers plus multiminor H antigenic barriers, or even to overcome xenogeneic antigen barriers, seems urgent to clinical transplantation. To achieve this goal, induction of a state of immunologic tolerance that includes a stable chimerism may be one practical approach (2).

Since a chimeric animal will accept almost all organs from a donor of bone marrow cells (3-5), various methods to establish chimerism for subsequent allo- or xenografting have been reported (6-10). Despite extensive studies, understanding of the cellular or molecular basis of immunologic tolerance in such chimeras remains enigmatic (11-16). Mechanisms that involve clonal deletion or inactivation, or active immunosuppression via suppressive lymphocytes, have been debated (11-16). In spite of the lack of understanding of the underlying process, a practical method for induction of immunologic tolerance represents a valid goal. If a long-lasting tolerant state that crosses the most forbidding barriers between donor and recipient could be achieved without hazard to the recipient, tissue and organ transplantation would be advanced.

We have reported a method of tolerance induction in adult mice that uses intravenous injection of 50-100 x 106 allogeneic donor-strain spleen cells followed, usually 2 d later, by 150-200 mg/kg of cyclophosphamide (CP) by the intraperitoneal route. Using this method, we have regularly established a minimal degree of mixed chimerism associated with apparently permanent allograft tolerance across a variety of H-2 incompatibilities.

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1 Abbreviations used in this paper: Ab, antibody; ALS, antilymphocyte serum; ANOVA, analysis of variance; BMC, bone marrow cells; CP, cyclophosphamide; DFR, delayed footpad reaction; H, histocompatibility; MST, mean survival time; SC, spleen cells.
of H barriers (17, 18, Mayumi, H., and R. A. Good, submitted for publication). However, it has not previously been possible to overcome regularly entire major H-2 plus multiminor H (fully allogeneic) barriers. Evidence suggested that this limitation could be attributed to development, during the induction of the tolerant state, of a population of cells that are resistant to CP and thus persist in a reactive rather than tolerant state (19, 20). From our further analysis, (Mayumi, H., and R. A. Good, submitted for publication), a less proliferative, more rapid maturation of reactive T cells was proposed to have occurred when the antigenic exposure was relatively small in proportion to a large population of persistently reactive cells in the recipient mice. Moreover, this less proliferative, more rapid maturation was found in preliminary studies to be eliminated by reducing the total number of reactive T cells in the recipient mice in preliminary experiments. In another study (21) we showed that stem cells capable of persistent replication represent a vital part of a regimen that induces long-lasting tolerance in the system we have developed.

The aim of the present study has been to develop a tolerance induction method that can regularly and specifically overcome fully allogeneic (entire H-2 plus multiminor H antigen) barriers in mature mice using allogeneic (tolerogenic) cells obtained from a single adult cadaver donor. To reduce immunocompetent T cells in recipient mice, mAbs against T cells or antibodies (Abs) versus different subpopulations of T cells were used. To enhance the tolerogenicity of spleen cells, bone marrow cells were added to the spleen cell inoculum to provide an enriched population of stem cells. When recipient mice were given 50-100 µg i.v. or i.p. of anti-Thy-1.2 Ab on day −1, 90 × 10^6 spleen cells plus 30 × 10^6 bone marrow cells from fully allogeneic donors intravenously on day 0, and then 200 mg/kg i.p. of CP on day 2, long-lasting skin allograft (grafted on day 15) tolerance that was associated with a minimal degree of mixed chimerism was regularly established in each of four fully allogeneic murine strain combinations. These manipulations could all be crowded together in a short interval and could be adapted to use materials from a single cadaver for production of the long-lasting tolerant state.

Materials and Methods

Animals. Inbred Thy-1.2+ female mice 8–10 wk old of BALB/cByJ (BALB;H-2d), C3H/HeJ (C3H; H-2b), C57BL/6J (B6; H-2b), and DBA/2J (DBA; H-2d) strains (the Jackson Laboratory, Bar Harbor, ME) were maintained in the University of South Florida Animal Research Center, St. Petersburg, FL. Mice were provided acidified water and were kept in sterilized cages on laminar air-flow racks.

Cell Preparations. Mice were killed by decapitation. The spleen, tibia, and femur were collected and kept on ice in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY). The spleen was disrupted in the medium by pressing spleen fragments between two glass slides. Bone marrow was flushed out using a 5-ml syringe with 25-G needle (Becton Dickinson & Co., Rutherford, NJ). Cell suspensions were filtered through cotton gauze and washed three times with the RPMI. Viable nucleated cells were counted and adjusted, usually to 120 × 10^6/ml. Viability of the cells was evaluated using trypan blue dye exclusion in the standard way.

Tolerance Induction. Purified anti-Thy 1.2 (rat IgG2a), anti-Lyt-1 (rat IgG2a), anti-Lyt-2 (rat IgG2a), and anti-L3T4 (rat IgG2b) mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). A given dose of antibody was injected intravenously or intraperitoneally as indicated. Usually, antibody was given 1 d before cell injection (on day −1). A given dose of spleen cells, bone marrow cells, or a mixture of these two kinds of cells from fully allogeneic donors was injected intravenously via the tail vein of recipient mice,
usually 2 d before CP treatment was given (Day of the cell injection = day 0). CP (Cytoxan; Bristol-Myers, Syracuse, NY) dissolved in PBS at a concentration of 20 mg/ml was administered in a dose of 200 mg/kg, usually 2 d after the cell injection (on day 2). In the assay to confirm optimal timing of antibody treatment and CP treatment, the timing of these treatments was varied as indicated (Table IV).

Skin Grafting. Using a procedure we have reported previously (22), skin grafting was carried out usually 13 d after CP treatment (on day 15). Graft beds, measuring 1 cm x 1 cm, were prepared on the right lateral thoracic wall. The panniculus carnosus was kept intact in the graft beds. Full-thickness square grafts, 1 cm x 1 cm, were sutured to the graft beds by eight interrupted sutures and were covered with protective tape. The first inspection of skin grafts was done 7 d after grafting, followed by daily inspections for 3 wk and weekly inspections thereafter. In the tolerant state, luxurious hair growth generally was observed, with the growth of hair being counterpoint to the normal hair growth and usually of different color than the normal hair of the recipient strain. The end-point of graft rejection was relatively easy to judge in both the acute state (within 30 d after grafting) and chronic state (later than 30 d after grafting), because the chronic rejection in the tolerant mice started with the loss of the hair and culminated in necrosis of the graft epithelium that was occasionally associated with ulcer formation. Therefore, in both acute and chronic rejection, the graft was considered to have been rejected when no normal epithelium could be found on the graft bed. Graft survival was expressed in days as mean survival time (MST) ± SD.

Assay of Delayed Footpad Reaction (DFR). DFR was elicited by injecting 0.05 ml of RPMI containing 10^7 C3H or BALB spleen cells irradiated with 2,000 rad using 6,000 Ci Cesium-137 (Mark I Model 68; JL Shepherd, Glendale, CA) into the right hind footpad of B6 mice. Into the left hind footpad, 0.05 ml of the RPMI alone was injected. Swelling was measured after 24 h with a dial-thickness gauge (Peacock, Kyoto, Japan). Net swelling was calculated by subtracting swelling in the left footpad from swelling in the right footpad. The first DFR challenge was performed 7 d after the CP treatment for tolerance induction (on day 9) to estimate the anamnestic immune status after the tolerance induction, as described previously (19, 20, 23). The second challenge was done 7 d after the first (on day 16), concurrent with intraperitoneal boosting for the assay of CTL activity. Therefore, the initial challenge was used not only to elicit DFR but also to immunize the mice for a second assay of DFR and for assays of CTL activity and antibody production.

Assay of CTL. Boosting for the CTL assay was carried out concurrently with the second DFR challenge (on day 16) in each mouse by injecting 0.2 ml of RPMI containing 10^7 2,000-rad irradiated C3H or BALB spleen cells into the peritoneal cavity with 5 ml of RPMI 3 d after intraperitoneal boosting with C3H or BALB spleen cells (on day 19). Peritoneal exudate cells that were nonadherent to glass were prepared by incubation of unfractionated peritoneal exudate cells in glass dishes for 1 h at 37°C, and then with gentle agitation by pipette. These glass nonadherent peritoneal exudate cells were adjusted to 10^7/ml or 5 x 10^6/ml in RPMI supplemented with 10% heat-inactivated FCS (KC Biological, Inc. Lenexa, KS) and were used as effector cells for the CTL assay. Contamination of the effector cells by macrophages was <10%.

Target cells were prepared by incubating B6, C3H, or BALB spleen cells at a concentration of 3 x 10^6/ml in the RPMI supplemented with 10% FCS containing 5 µg/ml of Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) for 2 d. 10^6 cells in 1 ml of the FCS were incubated with 200 Ci of ^51Cr (New England Nuclear, Boston, MA) for 1 h to label the target cells.

A standard ^51Cr-release assay was used in the humidified 5% CO₂ incubator for 4 h at 37°C. The assay was carried out in triplicate in a round-bottomed 96-well microplate (No. 25850; Corning Glass Works, Corning NY) in a total volume of 0.2 ml. The labeled target cells, 2 x 10^4 in 0.1 ml RPMI with 10% FCS, were added to effector cells, 1 x 10^6 and 0.5 x 10^6 in 0.1 ml. After the 4-h incubation, 0.1-ml of supernatant was harvested and counted using a γ counter (Multi-crystal gamma counter, Berthold, Wildbad, FRG). Percentage of specific release was calculated as follows: Percent specific lysis = 100 x [(experimental release - spontaneous release)/(maximal release - spontaneous release)]. The maximal release was obtained by adding 0.1 ml of 1% Triton X-100 (Thomas Scientific, Swedesboro, NJ) to 0.1 ml of target cells. The spontaneous release signifies the cpm released from target
cells incubated with the medium alone. The spontaneous release was <20% of the maximal release. All values represent the mean percentage specific $^{51}$Cr release of triplicate samples ± SD.

Assay of Antibody. Sera were collected and pooled from 10 mice when the mice were killed for the CTL assay (on day 17). The $^{51}$Cr-release assay with antibody was similar to the CTL assay described above (23), and was carried out in triplicate in microplates. The labeled cells, $2 \times 10^4$ in 0.1 ml of the RPMI with 10% FCS, were incubated with 0.1 ml of RPMI-diluted sera (×4, ×16) for 15 min at room temperature. After adding 0.02 ml of guinea pig complement (ICN Pharmaceuticals, Inc., Lisle, IL), the cells were incubated for 3 h more at 37°C in the humidified 5% CO$_2$ incubator. The supernatant was harvested and counted as well. Percentage of specific release was calculated using the equation described in the assay of CTL. The maximal release was obtained as well as the assay of CTL. The spontaneous release, which was <20% of the maximal release, was defined as the cpm released from the target cells incubated only with the medium plus complement. All values represent the mean percentage of triplicate samples ± SD.

Assay of Chimerism. The direct immunofluorescence method using FITC-conjugated anti-H-2K$^k$ or anti-H-2K$^d$D$^b$ mAb (Meiji, Tokyo, Japan) was used to stain spleen cells. Fluorescence-positive cells were measured with the FACS (EPICS C, Coulter Corporation, Hialeah, FL). Red blood cells among the spleen cells were destroyed by lysis with Tris-buffered 0.83% NH$_4$Cl before staining with the antibody. Red blood cells and dead lymphocytes were excluded by scatter. The chimerism of each mouse was assayed separately, with data expressed as the percentage of fluorescence-positive cells per 3,000 cells analyzed.

Statistics. When group data were parametric, one-way or two-way analysis of variance (ANOVA) was performed first. Only when the variance was significant was the Student's t-test performed (24). For multiple comparisons with both methods, the Bonferroni correction was used (25). $p$ values were calculated by means of a personal computer (PB-700; Casio, Tokyo, Japan). A $p$ value of <0.05 was taken to be significant.

Results

Long-Lasting Skin Allograft Tolerance Induced Across the Entire H-2 Plus Multiminor H Antigen Barriers. With the full protocol for inducing tolerance (Exp. 1, Table I; Group 11) a very long-lasting skin allograft tolerance to C3H(H-2$^k$) was produced in B6(H-2$^b$) mice. The protocol consists of injection of 100 µg i.p. of anti-Thy-1.2 antibody on day −1, an injection of $90 \times 10^6$ spleen cells plus $30 \times 10^6$ bone marrow cells from naive C3H mice on day 0, followed by an injection of 200 mg/kg i.p. of CP on day 2. The same results were obtained in other fully allogeneic donor→recipient combinations of B6→C3H (Exp. 2, Group 5), BALB(H-2$^d$)→B6 (Exp. 3, Group 5), and BALB→C3H (Exp. 4, Group 5).

Tolerance was not induced when the mAb and $90 \times 10^6$ spleen cells and $30 \times 10^6$ bone marrow cells alone, but not CP, were given (Exp 1, Group 8; Exp 2-4, Group 4). However, an appreciable level of tolerance, but not nearly so long-lasting a tolerance, was induced when $90 \times 10^6$ spleen cells plus $30 \times 10^6$ bone marrow cells and CP but no antibody, were given to the recipient (Exp. 1, Group 5; Exp. 2-4, Group 4). A more conventional method of tolerance induction used previously in our studies, i.e., allogeneic spleen cells ($120 \times 10^6$ rather than $100 \times 10^6$) followed in 2 d by CP, prolonged skin graft survival only slightly in the graft exchanges between these most disparate strain combinations (Exp. 1, Group 3; Exp. 2-4, Group 2).

Other groups treated in various ways were set up as controls in the donor-recipient combination of C3H→B6 (Exp. 1). Among these controls, neither CP alone (Group
2), 30 × 10^6 bone marrow cells plus CP (Group 4), antibody alone (Group 6), nor antibody plus CP (Group 7) prolonged graft survival. Moreover, treatments with antibody 120 × 10^6 spleen cells, plus CP (Group 9) induced only a slight degree of tolerance. Treatments with antibody, 30 × 10^6 bone marrow cells, and CP (Group 10) induced tolerance (MST = 53.0 d), but again this fell short of the more persistent tolerance induced by the full regimen (MST = 117.7 d), which in most mice produced a tolerant state that lasted as long as the experiments to test its persistence were carried out.

The specificity of the persistent tolerant state induced by this methodology was tested in the C3H→B6 and B6→C3H combinations. Tolerance induced in B6(H-2b) mice with antibody, 90 × 10^6 spleen cells plus 30 × 10^6 bone marrow cells from C3H(H-2k), and CP was specific to C3H since third-party skin from DBA(H-2d) was rejected in the tolerant mice with a normal tempo (Exp. 1, Group 13). In the reciprocal fully allogeneic combination of B6→C3H, the same results were obtained in the mice subjected to the full tolerance-inducing regimen (Group 7; Exp. 2).

**Dose-Response of anti-Thy-1.2 Antibody in Tolerance Induction in B6 Mice.** In the donor→recipient combination of C3H→B6, dose-response to anti-Thy-1.2 antibody was examined (Table II). Anti-Thy 1.2 antibody in a dose range of 12.5–100 μg was given intraperitoneally to the recipient B6 mice on day −1, followed by 90 × 10^6 spleen cells plus 30 × 10^6 bone marrow cells from C3H on day 0, and CP on day 2. The graft survival in the groups that received 12.5 μg (Group 3), 50 μg (Group 5), or 100 μg (Group 6) of antibody, C3H cells, and CP was significantly longer compared with that in a group that was given C3H cells plus CP, but not antibody (Group 2). These groups, however, did not show significant differences from one another. When 200 μg (0.8 ml) of the anti-Thy-1.2 antibody was injected intraperitoneally, the recipient B6 mice died. The quick death observed in this experiment was possibly attributable to sodium azide that was contained in the antibody preparation as preservative. Therefore, 100 μg (0.4 ml) was found to be the maximum dose of this antibody tolerated by the intraperitoneal route. Moreover, when the antibody was given intravenously, 50 μg (0.2 ml) was the maximal dose regularly tolerated. After conducting these experiments, dosages of 100 μg i.p. or 50 μg i.v. of the antibody were used in the tolerance-inducing regimen as indicated.

**Dose-Response of C3H Bone Marrow Cells in Tolerance Induction in B6 Mice.** In this experiment (Table III), dosages between 1 and 100 × 10^6 C3H bone marrow cells were injected into B6 mice together with or without 100 × 10^6 C3H spleen cells, and these cellular injections were administered with or without prior injection of 50 μg i.v. of anti-Thy 1.2 antibody.

As shown in Groups 3 and 5, tolerance induction was clearly dependent on the dose of bone marrow cells given. By contrast, when an inappropriately small dose of bone marrow cells had been given (Group 4), skin graft survival was further shortened as compared with untreated normal control mice (Group 1).

The tolerance induced with 1-100 × 10^6 bone marrow cells plus CP was made more profound when 100 × 10^6 spleen cells were added to the dosage of bone marrow cells given (Groups 6–8), but this effect only slightly prolonged skin graft survival. By pretreatment with 50 μg i.v. of anti-Thy 1.2 antibody, however, graft survival was greatly increased (Groups 9–11).
Table I
Long-Lasting Skin Allograft Tolerance Induced across Entire H-2 Plus Minor H Antigen Barriers

| Exp | Group | Recipient | Anti-Thy-1.2 antibody (100 μg; i.p., day -1) | Tolerogenic cells (×10^6, i.v., day 0) | CP (200 mg/kg; i.p., day 2) | Donor of skin graft, day 15 | Graft survival (days) | No. of mice | MST ± SD |
|-----|-------|-----------|--------------------------------------------|--------------------------------------|-----------------------------|-----------------------------|-----------------------|-------------|---------|
| 1   | 1     | -         | -                                          | -                                    | C3H                         | 9, 10, 10, 10, 11           | 15, 17, 17            | 5           | 10.0 ± 0.7 |
| 2   | -     | -         | -                                          | -                                    | C3H                         | 10, 10, 11, 11, 11          | 15, 17, 17            | 5           | 10.6 ± 0.5 |
| 3   | -     | C3H       | 120                                        | +                                    | C3H                         | 11, 12, 12, 13, 13, 14, 15  | 15, 17, 17            | 10          | 13.9 ± 2.1* |
| 4   | -     | C3H       | 30                                          | +                                    | C3H                         | 10, 11, 11, 11, 11          | 15, 17, 17            | 5           | 10.8 ± 0.4 |
| 5   | -     | C3H       | 90, 30                                      | +                                    | C3H                         | 14, 16, 19, 21, 24, 27, 31  | 32, 47, 47            | 10          | 27.8 ± 11.7* |
| 6   | +     | -         | -                                          | -                                    | C3H                         | 10, 10, 11, 11, 11          | 15, 17, 17            | 5           | 10.6 ± 0.5 |
| 7   | +     | -         | -                                          | -                                    | C3H                         | 10, 11, 11, 11, 12          | 15, 17, 17            | 5           | 11.0 ± 0.7 |
| 8   | +     | C3H       | 90, 30                                      | -                                    | C3H                         | 10, 10, 11, 11, 11          | 15, 17, 17            | 5           | 10.6 ± 0.5 |
| 9   | +     | C3H       | 120                                        | -                                    | C3H                         | 13, 14, 16, 16, 19          | 15, 17, 17            | 5           | 15.6 ± 2.3* |
| 10  | +     | C3H       | -                                          | 30                                    | C3H                         | 18, 39, 46, 81, 81          | 15, 17, 17            | 5           | 53.3 ± 27.6* |
| 11  | +     | C3H       | 90, 30                                      | +                                    | C3H                         | 32, 46, 81, 82, 95, 102, 102, 105, 116, 137, 137, 138, 144, 144, 151, 165, 222 | 19          | 117.7 ± 42.9* |
| 12  | -     | -         | -                                          | -                                    | DBA                         | 9, 10, 11, 11, 12, 13      | 15, 17, 17            | 5           | 11.0 ± 1.6 |
| 13  | +     | C3H       | 90, 30                                      | +                                    | DBA                         | 12, 12, 12, 14, 16, 16      | 15, 17, 17            | 5           | 13.2 ± 1.8* |
| 2   | 1     | -         | -                                          | -                                    | B6                          | 9, 9, 10, 10, 11            | 15, 17, 17            | 5           | 9.8 ± 0.8  |
| 2   | -     | B6        | 120                                         | +                                    | B6                          | 12, 14, 16, 17              | 15, 17, 17            | 4           | 14.8 ± 2.2* |
| 3   | -     | B6        | 90, 30                                      | +                                    | B6                          | 16, 21, 28, 52, 87          | 15, 17, 17            | 5           | 40.8 ± 29.3* |
| 4   | C3H   | +         | B6                                          | 90, 30                               | B6                          | 8, 9, 10, 11, 12            | 15, 17, 17            | 5           | 10.0 ± 1.6 |

continued
## Table I (continued)

| Exp | Group | Recipient | Anti-Thy-1.2 antibody (100 μg; i.p., day -1) | Tolerogenic cells (×10⁶, i.v., day 0) | Donor | SC | BMC | CP (200 mg/kg; i.p., day 2) | Donor of skin graft, day 15 | Graft survival (days) | No. of mice | MST ± SD |
|-----|-------|-----------|------------------------------------------|-----------------------------------|-------|----|-----|----------------------------|---------------------------|-----------------------|------------|---------|
| 5   | +     | B6        | 90 30                                   | +                                   | B6    | 66, 108, 108, 126, 136, 185, 185, 185 | 9         | >142.7 ± 44.4* |
| 6   | -     |           |                                          | -                                   | DBA   | 10, 10, 11, 11, 12 | 5         | 10.8 ± 0.8    |
| 7   | +     | B6        | 90 30                                   | +                                   | DBA   | 10, 12, 13, 13, 14 | 5         | 12.2 ± 1.5$  |
| 3   | 1     |           |                                          | +                                   | BALB  | 10, 11, 12, 12, 13 | 5         | 11.4 ± 1.1    |
| 2   | -     | BALB      | 120                                     | +                                   | BALB  | 13, 15, 17, 18, 20 | 5         | 16.6 ± 2.7*  |
| 3   | B6    |           |                                          | +                                   | BALB  | 19, 19, 25, 54, 110 | 5         | 45.4 ± 38.9* |
| 4   | +     | BALB      | 90 30                                   | +                                   | BALB  | 9, 9, 10, 10, 11  | 5         | 9.8 ± 0.8     |
| 5   | +     | BALB      | 90 30                                   | +                                   | BALB  | 19, 20, 82, 117, 131, >159, >159, >159, >159, >159, >159, >159 | 15       | >130.6 ± 50.2*|
| 4   | 1     |           |                                          | -                                   | BALB  | 11, 12, 12, 13, 13 | 5         | 12.2 ± 0.8    |
| 2   | -     | BALB      | 120                                     | +                                   | BALB  | 20, 21, 22, 24, 28 | 5         | 23.0 ± 3.2*  |
| 3   | C3H   |           |                                          | +                                   | BALB  | 46, 53, 53, 60, 60 | 5         | 54.4 ± 5.9*  |
| 4   | +     | BALB      | 90 30                                   | -                                   | BALB  | 8, 9, 9, 10, 12   | 5         | 9.6 ± 1.5     |
| 5   | +     | BALB      | 90 30                                   | +                                   | BALB  | 109, >165, >165, >165, >165, >165, >165, >165, >165, >165 | 8         | >158.0 ± 19.8*|

* p < 0.05 compared with Group 1 of each experiment (untreated controls) by the U-test.
<br>† Not significant compared with Group 12 of Exp. 1.
<br>$ Not significant compared with Group 6 of Exp. 2.
### Table II

*Dose-Response to Anti-Thy-1.2 Antibody in Tolerance Induction in B6 Mice*

| Group | Dose of i.p. anti-Thy-1.2 antibody on day -1 | C3H cells (9×10⁶ SC + 30×10⁶ BMC, i.v., on day 0) | CP (200 mg/kg, i.p., on day 2) | Survival of C3H skin graft (days)* | No. of mice | MST ± SD |
|-------|------------------------------------------|-------------------------------------------------|-------------------------------|----------------------------------|-------------|---------|
| 1     | -                                       | -                                               | -                             | 9, 10, 10, 11, 12               | 5           | 10.4 ± 1.1 |
| 2     | -                                       | +                                               | +                             | 15, 16, 17, 39, 53              | 5           | 28.0 ± 17.2 |
| 3     | 12.5                                    | +                                               | +                             | 46, 60, 74, 88, 109             | 5           | 75.4 ± 24.4f |
| 4     | 25.0                                    | +                                               | +                             | 21, 39, 53, 95, >158            | 5           | >73.2 ± 54.7 |
| 5     | 50.0                                    | +                                               | +                             | 46, 74, 88, 109, 109            | 5           | 85.2 ± 26.5f |
| 6     | 100.0                                   | +                                               | +                             | 60, 109, >158, >158, >158, >158 | 5           | >128.6 ± 43.8f |

* Grafting on day 15.

f *p* < 0.05 compared with Group 2 by the U-test.
TABLE III

Dose-Response to C3H Bone Marrow Cells in Tolerance Induction in B6 Mice

| Group | Anti-Thy-1.2 antibody (50 μg, i.v., on day -1) | C3H cells (10^6, i.v., on day 0) | CP (200 mg/kg, i.p., on day 2) | Survival of C3H skin graft (days)* | No. of mice | MST ± SD |
|-------|-----------------------------------------------|-------------------------------|--------------------------------|-----------------------------------|-------------|---------|
| 1     | -     | -     | -                             | 10, 10, 10, 11, 12            | 5           | 10.6 ± 0.9 |
| 2     | -     | 100   | -                             | 10, 11, 11, 12, 12            | 5           | 11.2 ± 0.8 |
| 3     | -     | -     | 1                             | 9, 9, 10, 11, 12             | 5           | 10.2 ± 1.3 |
| 4     | -     | -     | 10                            | 8, 8, 9, 9, 9                | 5           | 8.6 ± 0.5 |
| 5     | -     | -     | 100                           | 16, 17, 30, 54, >103         | 5           | >44.0 ± 36.4 |
| 6     | -     | 100   | 1                             | 11, 11, 11, 12, 15           | 5           | 12.0 ± 1.7 |
| 7     | -     | 100   | 10                            | 10, 13, 15, 19, 29           | 5           | 17.2 ± 7.4 |
| 8     | -     | 100   | 100                           | 16, 16, 17, 39, >103         | 5           | >38.2 ± 37.5 |
| 9     | +     | 100   | 1                             | 11, 13, 13, 29, 39           | 5           | 21.0 ± 12.4 |
| 10    | +     | 100   | 10                            | 23, 60, 67, 74, 88           | 5           | 62.4 ± 24.3 |
| 11    | +     | 100   | 100                           | 46, >102, >102, >102, >102   | 5           | >90.8 ± 25.0 |

* Grafting on day 15.
† p = 0.0180 compared with Group 1 by the U-test.
‡ p = 0.0180 compared with Group 2 by the U-test.

When the tolerogenicity of 100 × 10^6 spleen cells and 100 × 10^6 bone marrow cells was compared (Group 2 vs. Group 5), the bone marrow cells proved to be much more tolerogenic than the spleen cells.

Optimal Timing of Anti-Thy-1.2 Antibody and CP Treatments in Tolerance Induction in B6 Mice. In prior studies we (26, 27) and others (28–31) had tested the optimal timing of CP treatment to induce tolerance. This was shown to be 1–3 d after antigen stimulation. Under the influence of antibody treatment, however, the optimal timing of CP treatment may be changed (32). In this experiment, the anti-Thy-1.2 antibody was first used before the cell injection to achieve preparatory reduction of immunocompetent recipient T cells. Timing of anti-Thy-1.2 antibody treatment was also tested here (Table IV).

When 50 μg of anti-Thy 1.2 antibody on day -1, and 90 × 10^6 spleen cells plus 30 × 10^6 bone marrow cells from C3H donors were given on day 0 to B6 mice followed by CP treatment on day 0, 1, 2, 3, 4, or 5 (Groups 4–9), profound tolerance was induced only when CP was given on day 1, 2, or 3 (Groups 5–7).

When the antibody was given after the cell injection, i.e., C3H spleen cells plus bone marrow cells on day 0 followed by both antibody and CP on day 2 (Group 3), long-lasting tolerance was induced, as was the case for the group in which the antibody was given before the cell injection (Group 6).

Hereafter, the protocol for tolerance induction in the present experiments is always an initial antibody treatment on day -1, a second injection of tolerogenic cells on day 0, and a final CP treatment on day 2.

Complete and Specific Abrogation of Various Immune Responses in B6 Mice Made Tolerant of C3H with Anti-Thy-1.2 Antibody, C3H Spleen Cells, plus C3H Bone Marrow Cells, and then CP. Previously (19, 20, 23), we have shown that T cells mediating DFR are most resistant to tolerance induction with 50–100 × 10^6 fully allogeneic spleen cells plus 150–200 mg/kg CP, and are probably responsible for skin graft rejection (19, 20, 23) and development of graft-vs.-host disease (17). Moreover, the T cells remained
in a sensitized state after tolerance induction, probably because a less proliferative,
more rapid maturation occurred in some T cells during the 2-d interval between
antigen stimulation and CP treatment, while most of the reactive cells appeared to
be destroyed via an antigen-stimulated proliferating cell destruction by CP.

Using various immunological parameters, we examined and compared the tolerant
state in B6 mice induced conventionally with $120 \times 10^6$ C3H spleen cells and 200
mg CP, and that induced by our new method with 50 \mu g of anti-Thy-1.2 antibody
(intravenously), $90 \times 10^6$ C3H spleen cells plus $30 \times 10^6$ bone marrow cells, and
200 mg/kg CP.

As shown in Table V, the first challenge to elicit DFR 7 d after the CP treatment
was used to estimate the existence of memory T cells after tolerance induction. In
the B6 mice given $120 \times 10^6$ C3H spleen cells and CP (left column; Group 3), there
was a weak but significant level of footpad swelling as compared with negative con-
trols (left; Group 2). In the B6 mice made tolerant of C3H with the antibody,
$90 \times 10^6$ spleen cells plus $30 \times 10^6$ bone marrow cells, and CP, however, no residual
response could be found (left; Group 4). Using this first challenge for immuniza-
tion, a second challenge with allogeneic cells to elicit DFR was performed 7 d later.

In positive controls (right; Group 2), a strong response to C3H antigen was ob-
served. In the B6 mice treated with C3H spleen cells and CP (right; Group 3), a
moderate degree of response was achieved as compared with the negative controls
(Group 1). This response was weaker than that of the positive controls (Group 2).
In the B6 mice given antibody, spleen cells plus bone marrow cells, and then CP
(right; Group 4), a DFR was not detectable.

In these tolerant B6(H-2\textsuperscript{b}) mice given antibody, C3H(H-2\textsuperscript{b}) spleen cells plus bone
marrow cells, and CP, however, the DFR to a third-party antigen from BALB(H-
2\textsuperscript{d}) was normal (Group 7). Therefore, the tolerant state in the B6 mice was shown
to be tolerogen specific.

Assays of CTL activity (Table VI) and antibody production (Table VII) showed
similar patterns of responses. In the B6 mice given antibody, C3H spleen cells plus
### Table V

Delayed Hypersensitivity Footpad Reaction in B6 Mice Made Tolerant of C3H

| Group | Anti-Thy-1.2 antibody (50 μg; i.v., on day -1) | C3H cells (× 10⁶; i.v., on day 0) | CP (200 mg/kg; i.p., on day 2) | Challenge antigen (SC irradiated with 2,000 rad) | Footpad swelling (×0.1 mm) ± SD (n = 10) |
|-------|---------------------------------|--------------------------------|-------------------------------|------------------------------------------------|------------------------------------------|
|       | SC | BMC |                      |                                |                           | First challenge (on day 9) | Second challenge (on day 16) |
| 1     |    |     | 0.0001               |                                |                           | 0.8 ± 0.2                     |                           |
| 2     |    |     | 0.0001               |                                |                           | 1.0 ± 0.3                     | 2.5 ± 0.5                   |
| 3     |    |     | 0.0001               |                                |                           | 1.7 ± 0.7*                    | 1.5 ± 0.7¹                  |
| 4     |    |     | 0.0001               |                                |                           | 1.0 ± 0.3⁵                    | 0.8 ± 0.4²                  |

One-way ANOVA among Groups 1-4; *p value

| Group | Anti-Thy-1.2 antibody (50 μg; i.v., on day -1) | C3H cells (× 10⁶; i.v., on day 0) | CP (200 mg/kg; i.p., on day 2) | Challenge antigen (SC irradiated with 2,000 rad) | Footpad swelling (×0.1 mm) ± SD (n = 10) |
|-------|---------------------------------|--------------------------------|-------------------------------|------------------------------------------------|------------------------------------------|
|       | SC | BMC |                      |                                |                           | First challenge (on day 9) | Second challenge (on day 16) |
| 5     |    |     | 0.0001               | BAMB |                           | 0.6 ± 0.3                     | 2.6 ± 0.7                   |
| 6     |    |     | 0.0001               | BAMB |                           | 0.6 ± 0.5                     | 2.6 ± 0.7                   |
| 7     |    |     | 0.0001               | BAMB |                           | 1.0 ± 0.3                     | 3.0 ± 1.0¹                  |

One-way ANOVA among Groups 5-7; *p value

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SC, spleen cells.

* *p = 0.0052 compared with Group 2 by the t test.
† *p = 0.0053 compared with Group 1 by the t test.
§ Not significant compared with Group 2 by the t test.
¶ Not significant compared with Group 1 by the t test.
* *p < 0.0001 compared with Group 5, and not significant compared with Group 6 by the t test.
**TABLE VI**

*Cytotoxic T Lymphocyte Activity in B6 Mice Made Tolerant of C3H*

| Group | Anti-Thy-1.2 antibody (50 μg; i.v., on day -1) | C3H cells (x 10^6; i.v., on day 0) | CP (200 mg/kg; i.p., on day 2) | First challenge for DFR assay (on day 9) | Second challenge for DFR assay and i.p. boosting (on day 16) | CTL activity (% lysis ± SD; n = 3) against |
|-------|-----------------------------------------------|-------------------------------------|-----------------------------|---------------------------------|-------------------------------------------------|---------------------------------|
| 1     | -                                             | -                                   | -                          | C3H                             | 0.2 ± 1.6                                        | 0.8 ± 5.2                         |
| 2     | -                                             | -                                   | -                          | C3H                             | 0.5 ± 2.4                                        | 15.7 ± 3.5                        |
| 3     | -                                             | 120                                 | +                          | C3H                             | -0.1 ± 3.6                                      | 1.0 ± 4.0                         |
| 4     | +                                             | 30                                   | +                          | C3H                             | -0.5 ± 4.2                                      | -1.5 ± 2.0                         |

Two-way ANOVA among Groups 1-4; *p* value

<0.0001 (group), <0.0001 (target), and <0.0001 (interaction)

| 5     | -                                             | -                                   | -                          | BALB                            | -0.8 ± 4.3                                      | -4.6 ± 3.6                        |
| 6     | -                                             | -                                   | -                          | BALB                            | 39.0 ± 1.9                                      | 71.5 ± 13.0                       |
| 7     | +                                             | 90                                   | 30                         | BALB                            | -1.2 ± 1.1                                      | 67.5 ± 7.4                        |

Two-way ANOVA among Groups 5-7; *p* value

<0.0001 (group), <0.0001 (target), and <0.0001 (interaction)

* E/T cell ratio.
† *p* = 0.0035 compared with Group 1 by the *t* test.
‡ Not significant compared with Group 1 by the *t* test.
§ *p* < 0.0001 compared with Group 5 by the *t* test.
¶ *p* < 0.0001 compared with Group 5, but not significant compared with group 6 by the *t* test.
** *p* < 0.0001 compared with both Groups 5 and 6 by the *t* test.
### TABLE VII

**Antibody Activity in B6 Mice Made Tolerant of C3H**

| Group | Anti-Thy-1.2 antibody (50 µg; i.v.) | C3H cells (×10^6; i.v., on day 0) | CP (200 mg/kg; i.p. on day 2) | First challenge for DFR assay and i.p. boosting (on day 9) | Second challenge for DFR assay (on day 16) | B6 blast x2 | B6 blast x8 | C3H blast x32 | BALB blast x32* |
|-------|----------------------------------|----------------------------------|------------------------------|------------------------------------------------|----------------------------------|---------------|---------------|---------------|----------------|
| 1     | -                                | -                                |                              | C3H                                           | -0.7 ± 5.0                      | -0.5 ± 2.0    | 0.3 ± 3.1     | 0.2 ± 6.6     | -0.4 ± 3.5     | NT            | NT            |
| 2     | -                                | -                                |                              | C3H                                           | -0.3 ± 2.1                      | 26.4 ± 3.6    | 21.8 ± 4.2    | 3.4 ± 2.2     | 14.2 ± 1.8†    | NT            | NT            |
| 3     | -                                | 120                              |                              | C3H                                           | -0.3 ± 3.6                      | 2.2 ± 3.25    | 1.2 ± 0.6     | -0.2 ± 4.9    | 0.3 ± 5.8      | NT            | NT            |
| 4     | +                                | 90 30                            |                              | C3H                                           | -1.4 ± 4.0                      | -2.4 ± 4.85   | -1.3 ± 4.55   | -0.5 ± 8.0†   | 1.3 ± 3.3      | NT            | NT            |

Two-way ANOVA among Groups 1–4; p value
<0.0001 (group), <0.0005 (target), and <0.0001 (interaction)

| Group | Anti-Thy-1.2 antibody (50 µg; i.v.) | C3H cells (×10^6; i.v., on day 0) | CP (200 mg/kg; i.p. on day 2) | First challenge for DFR assay and i.p. boosting (on day 9) | Second challenge for DFR assay (on day 16) | B6 blast x2 | B6 blast x8 | C3H blast x32 | BALB blast x32* |
|-------|----------------------------------|----------------------------------|------------------------------|------------------------------------------------|----------------------------------|---------------|---------------|---------------|----------------|
| 5     | -                                | -                                |                              | BALB                                          | -1.3 ± 1.6                      | 0.2 ± 1.7     | NT            | NT            | 1.6 ± 1.9      | -2.3 ± 4.3    | -3.2 ± 3.8    |
| 6     | -                                | -                                |                              | BALB                                          | -0.8 ± 2.7                      | 27.1 ± 5.7†   | NT            | NT            | 50.4 ± 8.7     | 39.1 ± 7.0    | 32.7 ± 12.7   |
| 7     | +                                | 90 30                            |                              | BALB                                          | -0.9 ± 3.1                      | -0.4 ± 3.8    | NT            | NT            | 32.6 ± 8.9†    | 7.5 ± 8.3**   | 1.8 ± 6.6**   |

Two-way ANOVA among Groups 5–7; p value
<0.0001 (group), <0.0001 (target), and <0.0001 (interaction)

SC, spleen cells.

* Final concentration of the serum.
† p = 0.0006 compared with Group 1 by the t test.
‡ Not significant compared with Group 1 by the t test.
§ p < 0.0001 compared with Group 5 by the t test.
¶ p < 0.0001 and = 0.0105 compared with Groups 5 and 6, respectively.
** p < 0.0001 compared with Group 6, but not significant compared with Group 5.
C3H bone marrow cells, and then CP, CTL activity (Table VI, Group 4) and antibody production (Table VII, Group 4) against the tolerogen were completely abrogated. In the B6 mice given C3H spleen cells plus CP, both responses (Tables VI and VII; Groups 3) against the tolerogen also were almost completely abrogated, but a statistically insignificant level of weak response was recorded (second column).

Although cross-suppression could be demonstrated to some extent as compared with the positive controls (Tables VI and VII, Groups 6), CTL activity (Table VI, Group 7) and antibody production (Table VII, Group 7) against third-party BALB (H-2<sup>d</sup>) targets were observed in the B6 (H-2<sup>b</sup>) mice made tolerant of C3H (H-2<sup>k</sup>) with antibody, C3H spleen cells plus bone marrow cells, and then CP. Moreover, crossreactions of positive controls to third-party targets were observed to some extent in both the CTL assay (Table VI, Groups 2 and 6) and the antibody assay (Table VII, Groups 2 and 6). However, no response to syngeneic (B6) targets occurred in any of the groups examined (Tables VI and VII, Groups 1-7).

**Chimeric Analysis in B6 Mice Made Tolerant of C3H.** To analyze further the mechanisms of tolerance, the existence of mixed chimerism in the B6 mice made tolerant of C3H with 100 μg of anti-Thy 1.2 antibody (intraperitoneally), 90 x 10<sup>6</sup> C3H spleen cells plus 30 x 10<sup>6</sup> bone marrow cells, and then CP was examined (Table VIII). This seemed important because in other experiments in the series a minimal degree of mixed chimerism seemed a prerequisite for maintaining prolonged skin allograft tolerance in the system developed (17, 18).

In the B6(H-2<sup>b</sup>) mice made tolerant of C3H(H-2<sup>k</sup>) by the antibody, C3H spleen cells, bone marrow cells, plus CP regimen, a small number of H-2 K<sup>k</sup><sup>+</sup> cells of donor (C3H) origin were regularly detected among the recipient B6 spleen cells, and the proportion of H-2K<sup>bD</sup>b<sup>+</sup> cells of recipient origin was decreased slightly (Group 5) when the assay was done 1 mo after the tolerance induction. This minimal degree of chimerism in the tolerant B6 mice was also observed 2 mo after the tolerance induction, although the reciprocal reduction of H-2K<sup>bD</sup>b<sup>+</sup> cells of recipient origin was statistically insignificant (Group 5). In the B6 mice made tolerant

### Table VIII

**Chimeric Analysis in B6 Mice Made Tolerant of C3H**

| Group | Recipient* (pretreatment) | EPICS analysis: Percent positive cells ± SD (n = 5) |
|-------|---------------------------|---------------------------------------------------|
|       |                           | On day 33                                          | On day 62                                      |
|       |                           | H-2K<sup>b</sup> | H-2K<sup>bD</sup> | H-2K<sup>k</sup> | H-2K<sup>bD</sup> |
| 1     | C3H (untreated)           | 94.5 ± 2.2 | 0.7 ± 0.2 | 90.1 ± 1.3 | 1.0 ± 0.3 |
| 2     | B6 (untreated)            | 0.4 ± 0.1 | 94.5 ± 0.6 | 0.4 ± 0.1 | 83.2 ± 7.4 |
| 3     | B6 (CP alone)             | 0.3 ± 0.1 | 94.0 ± 0.6 | ND | ND |
| 4     | B6 (SC/CP)                | ND | ND | 0.6 ± 0.2<sup>†</sup> | 84.5 ± 4.4 |
| 5     | B6 (antibody/SC + BMC/CP) | 3.0 ± 0.8<sup>b</sup> | 89.3 ± 0.9<sup>b</sup> | 3.2 ± 1.4<sup>b</sup> | 75.8 ± 8.1 |
|       | One-way ANOVA among Groups 2-5; p value | <0.0001 | <0.0001 | = 0.0002 | = 0.1363 |

SC: spleen cells.

* The recipient B6 mice were made tolerant of C3H with 120 x 10<sup>6</sup> C3H spleen cells on day 0 and CP on day 2 (Group 4), or with 100 μg of anti-Thy-1.2 antibody (i.p.) on day −1, 90 x 10<sup>6</sup> C3H spleen cells plus 30 x 10<sup>6</sup> bone marrow cells on day 0, and CP on day 2 (Group 5).

<sup>†</sup> Not significant compared with Group 2 by the t test.

<sup>b</sup> p < 0.001 compared with Group 2 by the t test.
of C3H with $120 \times 10^6$ C3H spleen cells followed by CP, such stable chimerism was not established (Group 4).

**Tolerance Induced in B6 Mice with anti-Thy-1.2 Antibody, C3H Spleen Cells Plus C3H Bone Marrow Cells, and CP Is Attributable to Reduction of Effector Cells.** Transfer experiments were carried out to test the mechanisms of tolerance induced by the present method (Table IX). Tolerance to C3H was induced in B6 mice with 50 $\mu$g i.v. of anti-Thy-1.2 antibody, $90 \times 10^6$ C3H spleen cells plus $30 \times 10^6$ bone marrow cells, and then CP. 2 wk later, naive B6 mice and the tolerant B6 mice were grafted with C3H skin. $10^8$ million spleen cells and 0.3 ml of serum from the tolerant B6 mice were transferred into naive B6 mice that had been grafted on the same day with C3H skin (Group 3). Prolongation of C3H skin graft survival was not observed (Group 3), indicating that demonstrable tolerance was not transferred from the tolerant B6 into the naive B6 mice. On the other hand, when $100 \times 10^6$ naive B6 spleen cells were transferred into the tolerant B6 mice that had been grafted on the same day with C3H skin (Group 4), the C3H skin was rejected in a normal fashion in the tolerant B6 mice, indicating that tolerance was readily abrogated by the cell transfer. These results suggest that the main mechanism responsible for tolerance in this system is reduction of effector cells rather than a form of active suppression. These results, however, cannot exclude the existence of a weak level of suppressive cell and/or suppressive serum action in the tolerant mice.

**Tolerance Inducibility of Various Antibodies in B6 Mice.** Results from our in vivo (19, 20, 23, 26) and in vitro (33, 34) experiments and data presented herein (Table V), show that T cells mediating DFR and helper T cells, both of which are known to have L3T4 (CD4) markers (35), are the cells that are most resistant to tolerance induction. To further test this interpretation, various mAbs were used to induce tolerance in B6 mice (Table X). Although all of the antibodies used in these experiments were rat IgG antibody, differences of affinities may exist among the antibodies used; also, no evidence that the recipient B6 mice were saturated with the different antibodies used or equally suppressed with the amount of antibodies used was obtained. To analyze these data as objectively as possible, two different doses (50 $\mu$g in 0.2

### Table IX

| Group | Recipient (pretreatment) | Transferred cells and serum (on the day of skin grafting) | Survival of C3H skin graft (days) (grafting on day 15) | No. of mice | MST $\pm$ SD |
|-------|--------------------------|---------------------------------------------------------|-----------------------------------------------------|--------------|-------------|
| 1     | B6 (untreated)           | -                                                       | 9, 10, 11, 12, 13                                   | 5            | 11.0 $\pm$ 1.6 |
| 2     | B6 (tol. to C3H)*        | -                                                       | $>75, >75, >75, >75, >75$                           | 5            | $>75.0 \pm 0.04$ |
| 3     | B6 (untreated)           | $100 \times 10^6$ SC + 0.3 ml serum from B6 (tol. to C3H)* | 9, 10, 10, 12, 12                                   | 5            | 10.6 $\pm 1.35$ |
| 4     | B6 (tol. to C3H)*        | $100 \times 10^6$ SC from B6 (untreated)                | 10, 11, 11, 12, 12                                   | 5            | 11.2 $\pm 0.85$ |

* The B6 mice were given 50 $\mu$g of anti-Thy-1.2 antibody i.v. on day - 1, $90 \times 10^6$ C3H spleen cells plus $30 \times 10^6$ bone marrow cells i.v. on day 0, and 200 mg/kg CP i.p. on day 2.

† $p = 0.0271$ compared with Group 1 by the U-test.

§ Not significant compared with Group 1 by the U-test.
Table X
Tolerance Inducibility of Various mAbs in B6 Mice

| Group | Antibody (i.v., on day -1) | C3H cells (90 x 10⁶ SC + 30 x 10⁶ BMC; i.v., on day 0) | CP (200 mg/kg; i.p., on day 2) | Survival of C3H skin graft (days) (grafting on day 15) | No. of mice | MST ± SD |
|-------|-----------------------------|--------------------------------------------------------|---------------------------------|--------------------------------------------------------|-------------|---------|
| 1     | -                           | -                                                      | 9, 10, 10, 10, 10, 10, 11, 12, 13 | 10                                                      | 10          | 10.6 ± 1.2 |
| 2     | -                           | +                                                      | 9, 12, 13, 14, 15, 15, 16, 18, 20 | 10                                                      | 10          | 14.7 ± 3.1 |
| 3 a   | anti-Thy-1.2                 | 50.0 +                                                 | 15, 16, 22, 53, 53, 60, 74, 95, 102, >130 | 10                                                      | 4           | 56.3 ± 27.1 |
| b     | anti-Thy-1.2                 | 25.0 +                                                 | 18, 60, 67, 81                  | 10                                                      | 4           | 25.4 ± 36.4 |
| 4 a   | anti-Lyt-1                   | 50.0 +                                                 | 12, 12, 13, 14, 14, 15, 15, 16, >129 | 10                                                      | 5           | 17.0 ± 0.7 |
| b     | anti-Lyt-1                   | 25.0 +                                                 | 16, 17, 17, 17, 18              | 10                                                      | 5           | 25.8 ± 22.7 |
| 5 a   | anti-Lyt-2                   | 50.0 +                                                 | 13, 14, 15, 15, 15, 16, 17, 30, 38, 45 | 10                                                      | 5           | 25.8 ± 22.7 |
| b     | anti-Lyt-2                   | 25.0 +                                                 | 12, 13, 18, 20, 66              | 10                                                      | 5           | 25.8 ± 22.7 |
| 6 a   | anti-L3T4                    | 10.0 +                                                 | 16, 22, 46, 53, 60, 67, >74, 88, >130 | 9                                                       | 9           | 61.8 ± 34.5 |
| b     | anti-L3T4                    | 5.0 +                                                  | 13, 14, 16, 46, 53              | 5                                                       | 5           | 28.4 ± 19.4 |
| c     | anti-L3T4                    | 2.5 +                                                  | 13, 18, 18, 46                  | 5                                                       | 5           | 22.8 ± 13.2 |
| 7     | anti-Lyt-2                   | 50.0 +                                                 | 23, 26, >74, >74, >74           | 5                                                       | 5           | >54.2 ± 27.1 |

* p < 0.01 compared with Group 2 by the U-test.
† p = 0.0533 compared with Group 2 by the U-test.
‡ Not significant compared with Group 2 by the U-test.
¶ Not significant compared with Group 6-a by the U-test.
ml and 25 μg in 0.1 ml) were used. For anti-L3T4 antibody, the doses of 10 μg in 0.2 ml, 5 μg in 0.1 ml, and 2.5 μg in 0.05 ml were used. These antibodies were given intravenously on day -1, followed by 90 x 10^6 C3H spleen cells plus 30 x 10^6 bone marrow cells on day 0, and CP on day 2.

The longest survival was achieved when the B6 mice were treated with 50 μg or 25 μg of anti-Thy-1.2 antibody (Groups 3 a and b) or 10 μg of anti-L3T4 antibody (Group 6 a). Both anti-Lyt-1 antibody and anti-Lyt-2 antibody appeared to have some influence to facilitate tolerance induction, but the effect of the latter two antibodies was slight (Groups 4 and 5). The addition of 50 μg of anti-Lyt-2 antibody to 10 μg of anti-L3T4 antibody may have exerted an additive effect, but this influence was not impressive (Group 7), even though the number of mice in this group was quite small.

Discussion

Using three treatment components administered within a 3-d period, namely anti-Thy-1.2 antibody (or anti-L3T4 antibody), allogeneic spleen cells plus bone marrow cells, and CP, we regularly induced very long lasting skin allograft tolerance across fully allogeneic (all known H-2 components plus multiminor H) antigen barriers in each of four murine combinations examined (Table I). Since the tolerogenic cell doses used can be obtained from a single donor mouse, and since the total dose of 200 mg/kg CP used was quite tolerable for mice and did not require subsequent hematopoietic reconstruction, the strategy used can be considered to be one that is possibly adaptable to other species, e.g., lower primates and even man. Indeed, a comparable dose of CP given over 3 d is tolerated by monkeys (36). A maximal use of anti-T cell or anti-CD4 antibody before (Tables I-X), during, and/or after (Table IV) tolerance induction might further reduce the amount of CP needed. This issue should be examined experimentally first in lower primates.

The clues that led to the development of the present potentially practical protocol for regularly inducing long-lasting tolerance during adult life were derived from our earlier studies (17-23, 26, 27). First we showed that tolerance to a large allogeneic tumor inoculum, but not to a small dose of tumor cells or to a skin allograft, can be achieved with 50-100 x 10^6 viable fully allogeneic spleen cells followed by 150-200 mg/kg CP (19, 20, 23, 26). The tolerant state achieved by this combined treatment with spleen cells plus CP that uses the spleen cells as antigenic stimulation to induce cell proliferation permits later destruction of the proliferating cells with CP. Although it was a form of split tolerance, it was induced across fully allogeneic antigen barriers in which resistance to more complete tolerance appeared to be attributable to a relatively small proportion of less proliferative T cell clones or to a rapid maturation of T cells to a CP-resistant state without their becoming vulnerable as when proliferation occurs (19, 20, 23). Since permanent skin allograft tolerance could regularly be induced across multiminor H antigen disparities (27, 37) and also quite easily across the H-Y antigen barrier (38) with 150-200 mg/kg CP, the dose of CP was considered to be sufficient. To increase the dose of the spleen cells to >100 x 10^6 was considered impractical because this appeared to be the maximal dose of spleen cells that could be collected from a single donor mouse at one time. Therefore, attempts to change the less proliferative mode of immune response into a more uniformly proliferative mode that might render the reacting cells sus-
ceptible to CP were made by a variety of methods (23, 39, 40). However, permanent or very long lasting skin allograft tolerance across fully disparate MHC plus multiminor allogeneic barriers was not achieved by the manipulations (23, 39, 40).

Studies to test sensitivity to tolerance induction using congenic strains of mice (Mayumi, H., and R. A. Good, submitted for publication) revealed that resistance (or less proliferative maturation of reactive T cells) to tolerance induction across fully allogeneic barriers is generated by cumulative influences on the recipient T cells that are reactive against donor class I, class II, or multiminor H antigens. Moreover, induction of semipermanent skin graft tolerance with normal hair growth across fully allogeneic barriers was achieved using a two-step induction method in which the frequency of recipient T cells reactive at any one time was reduced by using stimulating spleen cells from congenic mice as a first-step tolerogen (Mayumi, H., and R. A. Good, submitted for publication). This study suggested that an anti-T cell antibody might be used as a preparatory step to reduce the reactive T cells in the recipient. Furthermore, in another series of studies, it appeared that stem cells contained in the tolerogenic spleen cell inoculum might play a critical role in establishing a stable tolerant state (21). Therefore, bone marrow cells were added to the spleen cell inoculum in an effort to boost tolerogenicity by boosting the dose of stem cells in the present study.

As shown in Table 1, a very long lasting and often impressively persistent tolerant state was induced only when all of the three components of the treatment were used: first antibody, then spleen cells plus bone marrow cells, and finally a single large dose of CP. However, spleen cells plus bone marrow cells and CP (Exp. 1, Group 5; Exps. 2–4, Group 3), or antibody, bone marrow cells, and CP (Exp. 1, Group 10) induced a significant degree of tolerance without the mAb. This finding suggested that the bone marrow cells contribute significantly to the induction of the tolerant state. The tolerogenic capability of bone marrow cells was examined both qualitatively and quantitatively (Table III) and was found to make a greater contribution to long-lasting tolerance than did the viability of the spleen cells. There seemed little doubt that anti-Thy-1.2 antibody contributed to tolerance induction, as was shown in various fully allogeneic combinations (Tables I and III), and this point was confirmed quantitatively (Tables II and X). The role of spleen cells as an effective source of antigen that can evoke proliferation of reactive T cells (21) appeared to be confirmed in the present study by comparing Groups 4 and 5 (Table I, Exp. 1), Groups 10 and 11 (Table I, Exp. 1), or Groups 3–5 and Groups 6–8 (Table III).

Other possible explanations for tolerance induction in the present study were dismissed by the several control groups included in Table I. For example, the tolerance might have been induced simply because of the increment in the number of tolerogenic spleen cells from $100 \times 10^6$ (17, 23, Mayumi, H., and R. A. Good, submitted for publication) to $120 \times 10^6$. But this was found not to be the case (Exp. 1, Group 3; Exps. 2–4, Group 2). With CP alone (Exp. 1, Group 2) or with antibody alone (Exp. 1, Group 6), tolerance was not produced. The possibility that the influence of the rat IgG (anti-Thy-1.2) antibody was prolonged because of the tolerance induction against the antibody itself with CP (41) could be excluded (Exp. 1, Group 7). Moreover, the possibility that the tolerance was induced with antibody plus lymphoid cells alone but not with CP (6, 42, 43) was excluded in all of the combinations examined (Exp. 1, Group 8; Exps. 2–4, Group 4).
Optimal timing of CP treatment was reevaluated in view of Wood and Monaco's report (32) that the optimal timing of CP treatment in their protocol of antilymphocyte serum injection, skin grafting, and then bone marrow injection was 5 d after bone marrow injection. In another series (38), we have shown that when the number of reactive T cells is small, the optimal timing of CP treatment tends to be more delayed. Since on theoretical grounds we used the antibody to reduce the number of reactive T cells in the recipients, one might have expected some delay of optimal time for the CP treatment. Contrary to our expectation, however, the optimal timing of the CP treatment was found to be 1–3 d after the cell injection, just as had been found in previous analyses (26, 27, 33). It may not be surprising that the anti-Thy-1.2 antibody was effective even when the antibody had been given concurrently with CP; i.e., 2 d after the cell injection. This observation suggests the possibility that the antibody may exert an action to destroy T cells that usually remain after tolerance induction with spleen cells plus CP (19, 20, 23, and Table V).

In the B6 mice made tolerant of C3H with anti-Thy-1.2 antibody, C3H spleen cells plus bone marrow cells, and then CP, a minimal degree of mixed chimerism was established (Table VIII), and all immune parameters representative of specific immunity that were examined appeared to be completely abrogated (Tables V-VII). These results are consistent with the observation of the very long skin graft survival in the tolerant mice (Tables I-IV, IX, and X). By contrast, in the B6 mice made tolerant of C3H by injections of C3H spleen cells plus CP, a weak level of DFR remained after tolerance induction (Table V), even though CTL activity and antibody production were quite impressively abrogated (Tables VI and VII). In this group, neither mixed chimerism (Table VIII) nor apparently permanent skin allograft tolerance (Tables I and III) was established.

We added bone marrow cells to increase the number of stem cells and to facilitate the establishment of chimerism as discussed above. However, normal adult bone marrow is known to contain natural suppressor cells (11, 44) or natural suppressor cell activities (45), and also to contain what have been called veto cells (46). It is possible that such populations may have contributed to the establishment of the chimeric state. Moreover, in spite of the establishment of a mixed chimerism, there was no sign of graft-vs.-host disease in the tolerant recipient mice in the present system. To establish and maintain this state of stable mixed chimerism, the role of the anti-Thy-1.2 antibody appeared to be crucial, as was a role for CP presumably to destroy both recipient T cells reactive against donor antigens and donor T cells reactive against recipient antigens, because eventually lethal graft-vs.-host disease occurred in B6 mice (10/10) that were given anti-Thy-1.2 antibody, AKR/J (H-2k; Thy-1.1) spleen cells, and then CP (Mayumi, H., and R. A. Good, unpublished data), suggesting that the anti-T cell antibody suppressed not only the recipient T cells but also the donor T cells in the present system.

In the transfer experiments reported here (Table IX), we could not detect any strong suppressive influences that might prolong skin allograft survival directly (Group 3). Moreover, the tolerant state was readily abrogated by transferring syngeneic naive spleen cells to the tolerant recipients (Group 4). These results suggest that the mechanism of tolerance induction and maintenance in the present scheme is largely attributable to reduction of effector T cells that are reactive against the tolerogen, whether the mechanism to generate this state involves active clonal deletion, clonal inactiva-
Cyclophosphamide-induced tolerance in adult mice

The actual cells that mediate graft rejection in mice are a subject of some debate. Loveland et al. (47) reported that Lyt-1+ cells mediate skin graft rejection (47). However, extensive studies using congenic strains of mice in both skin graft transplantation (48-51) and bone marrow transplantation (51-54) have clarified that rejection of class I and class II antigens is largely mediated by Lyt-2+ and L3T4+ T cells, respectively, while rejection of multiminor H antigens may be mediated by either of the two major T cell subsets, depending on the strain combinations used. Our previous (17,19,20,23,26,34,40) and present (Table V) studies consistently appear to indicate that T cells (probably L3T4+) that also can mediate DFR are primarily responsible for rejection of skin allografts and for causing graft-vs.-host reactions in fully allogeneic murine combinations. This conclusion seems to be strongly supported by our final experiment (Table X). Despite a smaller dose of the L3T4 (CD4) antibody used, this antibody showed a potent action to facilitate tolerance induction (Group 6). This result to date remains limited to the C3H→B6 combination. To our surprise, however, not only anti-Lyt-2(CD8) antibody but also anti-Lyt-1(CD5) antibody was less effective than the anti-L3T4 antibody in promoting tolerance induction. Further studies to permit qualitative conclusions applicable to other mouse strains are surely required.

A number of other investigators have achieved an apparent tolerant state sufficient to permit very long lasting skin allograft tolerance in mice just as described herein. The impressive initial results with total nodal irradiation plus bone marrow transplantation have not been readily extended to larger animals and may have been difficult to generalize in mice; further, the mechanism of tolerance in this instance apparently involves a strong nonspecific suppressive action of lymphoid cells and thus seems very different from the tolerant state described herein (7, 55). Wood and Monaco have been struggling for some time to develop a system of immunological tolerance that might ultimately be applied to larger animals and even to man (32, 56, 57). In the first system, they have described tolerance that was achieved by treatment with antilymphocyte serum (ALS). This method used the donor-recipient combination of A→C3H, which represents a partial match at the MHC. In addition, (C3H × A)F1 spleen cells (a haploidentical system) were used in large numbers to facilitate tolerance induction. Thus for several reasons, not the least of which are the use of adult thymectomy and the employment of F1 cells, their strategy could not be the basis for development of a method that might ultimately be clinically useful. The next model these investigators explored was based on a method used by Lance and Medawar (42). In this model, CP was used both before or after the bone marrow cell treatment (32), and when CP was given 1-7 d after the bone marrow cells, graft survival was significantly prolonged. A major difference between this ap-
proach and the method we used is in the timing of the test skin graft. Monaco et al. showed that if skin grafting was done after bone marrow cell injection, tolerance was not induced (56, 57). In this system, the allogeneic skin graft itself apparently contributed in some crucial way to the tolerance induction. In the system we have described herein, once tolerance is induced with anti-Thy-1.2 antibody then spleen cells plus bone marrow cells followed by CP, timing of the test skin allograft is not of importance, and the graft appears to play no role in the induction of the tolerant state. Thus, it is clear that the difference between the protocol of Wood and Monaco and that described herein is not only that we have used an mAb rather than ALS, but also that we have used a combination of manipulations that do not depend on the organ or tissue graft used for a contribution to the tolerance induction.

Relatively recent investigations by Ildstad et al. (51, 58, 59) in which a stable mixed chimerism is established by a combination of bone marrow transplants of T cell-purged syngeneic plus T cell-purged allogeneic marrow have been shown to induce a tolerant state without producing graft-vs.-host reaction. This method permits development of a tolerant state that, like the one we have produced herein, is associated with a stable mixed chimerism and long-lasting tolerance that can bridge differences all across the entire MHC. Thus far, however, this method has not permitted skin transplantation across entire H-2 plus multiminor barriers (C3H→B10) without chronic skin graft rejection. Although the difficulty with chronic skin graft rejection was attributed to skin specific alloimmunity (60, 61), skin graft rejection did not appear to be a problem after production of tolerance by the method described herein.

Bone marrow transplantation after which the animals are maintained in a specific pathogen-free environment or, in separate experiments, in a clean but conventional environment, has also been used to produce an impressive state of tolerance that permits regular allotransplantation of skin (62, 63), liver (64), and even pancreas (65) without rejection. In this model recipients have been shown to be tolerant of donor, tolerant of recipient, and fully reactive to third-party in T cell-mediated responses. However, mice tolerized by bone marrow transplantation in some systems have exhibited an immunodeficiency leading to high morbidity and high mortality (66, 67) when the mice are moved from laminar air flow gnotobiotic environments to more conventional environments. The BMT tolerance model has also been associated with demonstrable immunologic deficits that have in some systems limited fully vigorous immunologic responses in the recipients. This deficit has been shown to be attributable to deficient interactions of donor and recipient cells (62, 63, 68). Although this deficit is not as devastating as was originally presumed (62, 69), it represents a limitation nonetheless (70). The advantage of the method of tolerance induction we have described herein and also an alternative method described previously by Ildstad et al. (51, 58, 59) is the possibility that either of these methods might be adaptable to tolerance induction in lower primates or even ultimately in humans. Future attempts to adapt tolerance-producing strategies to lower primates and to humans based on modifications of one of these two different models now seem much in order.

Summary
A new method of cyclophosphamide (CP)-induced skin allograft tolerance in mice that can regularly overcome fully allogeneic (major H-2 plus non-H-2) antigen barriers in mice has been established. The components of the method are intravenous
or intraperitoneal administration of 50-100 μg of anti-Thy-1.2 mAb on day -1, intravenous injection of 90 × 10^6 allogeneic spleen cells mixed with 30 × 10^6 allogeneic bone marrow cells from the same donor on day 0, and intraperitoneal injection of 200 mg/kg CP on day 2. In each of four fully allogeneic donor → recipient combinations, including C3H/HeJ (C3H; H-2^k)→C57BL/6J (B6; H-2^b), B6→C3H, BALB/cByj (BALB; H-2^d)→B6, and BALB→C3H, long-lasting survival of skin allografts was induced in most of the recipient mice. The specific tolerant state induced was dependent on the doses of the antibody and bone marrow cells used. The optimal timing of CP treatment to induce tolerance was found to be 1-3 d after the stimulating cell injection. Treatment with the anti-Thy-1.2 antibody together with CP on day 2 after the cell injection on day 0 also induced profound tolerance. In the B6 mice made tolerant of C3H with antibody, C3H spleen cells plus C3H bone marrow cells, and then CP, a minimal degree of stable mixed chimerism was established and the antitolerogen (C3H) immune responses examined here, including delayed footpad reaction (DFR), CTL activity, and capacity for antibody production against donor-strain antigens were abrogated in a tolerogen-specific manner. From cell transfer experiments, the mechanism of tolerance could be largely attributed to reduction of effector T cells reactive against the tolerogen, and strong suppressive influences that might prolong skin allograft survival directly were not detected in the tolerant mice. Moreover, pretreatment with anti-Thy-1.2 antibody or anti-L3T4 (CD4) antibody was more effective than pretreatment with anti-Lyt-1 (CD5) antibody or anti-Lyt-2 (CD8) antibody as an initial step in tolerance induction.

These results suggest that permanent tolerance to fully allogeneic skin grafts may be induced because antibody given before the stimulating cell injection reduces the number of reactive T cells in the recipient mice. This antibody treatment may facilitate an antigen-stimulated destruction of responding and thus proliferating cells with CP by preventing a possibly less proliferative, more rapid maturation of reactive T cells or by destroying residual effector T cells. The injection of bone marrow cells mixed with spleen cells appears to have facilitated maintenance of the tolerant state by establishing a state of stable mixed chimerism in the tolerant mice.

This methodology, which induces within a very short interval a long-lasting tolerant state in immunologically mature mice, may possibly be adaptable to production of full immunological tolerance across major plus minor histocompatibility barriers in other mature mammals, e.g., lower primates and even humans. If this proves to be the case, it could greatly facilitate organ transplantation without the need to constantly depress immunologic reactivities, as is now needed in the practice of organ and tissue transplantation in humans.

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