T suppressor (Ts) cells have been shown to play an important role in the regulation of a variety of immune responses. In many of these systems of immune regulation, it has been demonstrated that the activation and/or effector function of these Ts cells is genetically restricted. Thus, for example, the interaction of Ts cells either with other T cells in a suppressive network or with the cells that are the targets of suppression has been reported by several laboratories to be restricted by genes in the I-A subregion (1-11) of the murine major histocompatibility complex (MHC). In other circumstances, Ts cell function has also been found to be restricted by products of other MHC genes (12-15) or of non-MHC-linked genes (16). The presence of such genetic restrictions in cell interaction has provided an important instrument for the study of the mechanisms of Ts cell activation and function.

It has recently been demonstrated that two distinct Ts cell pathways function to regulate the MHC-restricted and carrier-hapten-linked IgG responses of Lyb-5− B cells (17-21). In one pathway, carrier-primed Lyt-1−2− Ts cells are activated in an antigen-specific manner by in vitro reexposure to the priming antigen. After this specific activation, these Lyt-1−2− Ts cells are able to suppress IgG responses in an antigen-nonspecific manner. This suppression requires the participation of unprimed Lyt-1−2+ T cells, and is effective in both the early and late phases of antibody responses. A second suppressor pathway requires the antigen-specific activation of primed Lyt-1−2+ Ts cells and is highly carrier specific in its effector function, in contrast to the nonspecific effector function of Lyt-1−2− Ts cells. This Lyt-1−2+ Ts population appears to act without requirement for additional T cell populations, and is effective only early in the course of the antibody response. Thus, it appears that two Ts cell populations may function through distinct mechanisms to regulate the generation of IgG Lyb-5− B cell responses.

In order to further analyze the cell/cell interactions which are involved in the Ts cell regulation of IgG responses by Lyb-5− B cells, the existence of genetically restricted cellular interactions was evaluated in the present studies. It was demonstrated that
accessory cells as well as antigen-primed T cells are required for the in vitro activation of both Lyt-1+2- and Lyt-1-2+ Ts cells. Moreover, T cell recognition of I-A-encoded determinants on these accessory cells is required for in vitro activation of Ts cells, and this interaction determines the subsequent I-A restriction of Ts cell effector activity.

It was found that the I-A restriction in suppressor function reflects neither an MHC restriction in the interaction of Ts cells with T helper (Th) cells, nor the simple consequence of Ts cell recognition of I-A products on B cells or accessory cells. Rather, the Ts cell function characterized here appears to act in a unique and highly specific manner to interfere with the MHC-restricted interaction between Th cells and the B cells and/or accessory cells cooperating in these antibody responses.

Materials and Methods

**Animals.** C57BL/6 (B6), C57BL/10SgSn (B10), B10.A, B10.BR, (B10 × B10.A)F1, (B10 × B10.BR)F1, (B6 × C3H/He)F1, (B6 × A/J)F1, (B6AF1) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. A.TH, A.TL, (A.TH × A.TL)F1, B10.A(3R), B10.A(4R), and B10.A(5R) mice were provided by Dr. D. H. Sachs, National Institutes of Health.

**Chimeras.** Chimeras used in this study were prepared as previously described (22) by the transfer of 15 × 10⁶ T cell-depleted bone marrow cells into lethally irradiated (900-950 rad) recipients, and are designated as bone marrow donor → irradiated recipient. Chimeras were immunized no earlier than 8 wk after irradiation and reconstitution. The chimera spleen cells used in these studies were typed by indirect immunofluorescence and were of donor origin without detectable (<5%) host contamination.

**Antigen.** Keyhole limpet hemocyanin (KLH) (lot 730192; Calbiochem-Behring Corp., San Diego, CA) and fowl gamma globulin (FGG) (Cappel Laboratories, Cochranville, PA) were conjugated with 2,4,6-trinitrobenzene sulfonate (Pierce Chemical Co., Rockford, IL) as previously described (18). The degrees of substitution were 20 trinitrophenyl (TNP) residues/100,000 daltons KLH (TNP-KLH) and 9 TNP residues/100,000 daltons FGG (TNP-FGG).

**Antisera.** Monoclonal hybridoma anti-Lyt-l.2 antiserum (lot FPB-031) and anti-Lyt-2.2 antiserum (lot FPA-179) were purchased from New England Nuclear, Boston, MA, and used as cytotoxic reagents at final dilutions of 1:800 and 1:3200, respectively, on 1 × 10⁵ cells/ml as previously described (21). Monoclonal anti-Thy-1.2 antiserum was a generous gift from Dr. P. Lake (Georgetown University, Washington DC). Monoclonal anti-I-A<sup>+</sup> reagent was prepared as a culture supernatant of the anti-I-A<sup>+</sup> hybridoma designated 10-2.16, described by Oi et al. (23), which was obtained from the Cell Distribution Center of the Salk Institute, La Jolla, CA.

**Immunization.** Mice (except parentA → (A × B)F1 chimeras) were immunized with 100 μg of KLH, FGG, or TNP-KLH in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) intraperitoneally 3-8 wk before use. ParentA → (A × B)F1 chimeras were immunized with 5 × 10⁵ KLH-pulsed (A × B)F1 3,000 rad-irradiated spleen cells (see below) in Bordetella pertussis antigen (Difco Laboratories) intraperitoneally 3-8 wk before use.

**Preparation of Cells**

**T cells.** KLH or FGG-primed T cells were obtained as spleen cells nonadherent to antimouse immunoglobulin-coated plastic dishes as described previously (19). These populations were then used as Th cells or as a precursor population for the generation of Ts cells (see below).

**(B + ACCESSORY) cells.** (B + accessory) cells were prepared by depleting TNP-KLH-primed spleen cells of T cells by treatment with a T cell-specific cytotoxic rabbit anti-mouse brain serum (RAMB) + complement (C) (24).

**ACCESSORY CELLS.** Unprimed spleen cells were used as a source of accessory cells following 3,000 rad irradiation. In some experiments, spleen cells were treated with RAMB + C to deplete T cells. To immunize parentA → (A × B)F1 chimeras, accessory cells were incubated at a density of 1 × 10⁶ cells/ml in culture medium containing 100 μg/ml KLH at 37°C for 4 h and washed extensively, then used as antigen-pulsed accessory cells.
Suppressor cell induction in vitro. KLH-primed T cells were passed over Sephadex G-10 columns twice to deplete accessory cells (24, 25). 3 x 10^7 accessory cell-depleted KLH-primed T cells were suspended in 5 ml of 10% fetal calf serum containing RPMI 1640 medium supplemented as previously described (19) in the presence or absence of additional accessory cells (2 x 10^6/ml) and of 100 µg/ml KLH. Cells were cultured (precultured) in tissue culture dishes (Costar 3506; Costar, Cambridge, MA) for 3 d at 37°C in 5% CO2 humidified air atmosphere, harvested, washed extensively, and tested for their suppressive activities. In some experiments as indicated in the text, T cells were used without passing over Sephadex G-10 columns.

Assay of Suppressive Activity. 1 x 10^6 (or, as indicated in the text, titrated numbers of) precultured T cells were assayed for suppressive activity by the addition to responding cell mixtures of 3 x 10^6 TNP-primed (B + accessory) cells co-cultured with 1 x 10^6 KLH- or FGG-primed Th cells. In some experiments, 3 x 10^6 TNP-KLH-primed untreated spleen cells syngeneic to Ts cells were used as responding cells. Cultures were carried out as previously described (19). Cells were harvested and assayed for TNP-specific hemolytic plaque-forming cells (PFC).

PFC Assay. Sheep erythrocytes (SRBC) were conjugated with TNP (TNP-SRBC) and direct PFC (IgM) as well as total PFC (facilitated by rabbit anti-MIgG) were assayed on these conjugated target cells (19). IgG PFC were calculated as total PFC minus direct PFC. All points shown represent the arithmetic means of triplicate cultures.

Results

Accessory Cells Are Required for the Activation of Ts Cells In Vitro. Two Ts cell pathways were recently characterized that function to selectively regulate the in vitro IgG responses of Lyb-5^- B cells (17). It was demonstrated that the activation of each of these Ts populations required both in vivo priming and subsequent in vitro stimulation with specific antigen. To further analyze the cellular interactions required for activation of these Ts cells in vitro, it was first determined whether the presence of accessory cells as well as T cells was required for the in vitro induction of Ts activity. KLH-primed B10 T cells were depleted of accessory cells by passage over Sephadex G-10 columns and were then cultured (precultured) for 3 d in the presence or absence of added unprimed B10 accessory cells and in the presence or absence of 100 µg/ml KLH or FGG. Precultured cells were harvested, washed and tested for their suppressive activities on the responses to TNP-KLH by TNP-KLH-primed B10 spleen cells (Table I). KLH-primed B10 T cells that had been precultured with 100 µg/ml KLH in the presence of added B10 accessory cells significantly suppressed the response of primed B10 spleen cells to TNP-KLH (Table I). In contrast, T cells that had been precultured either without added accessory cells or without the relevant antigen to which they had been primed in vivo failed to suppress these in vitro responses. In addition, the suppressive activity generated under these conditions was completely eliminated by treatment with anti-Thy-1.2 + C. These results demonstrated that the presence of both accessory cells and specific antigen is required for the in vitro activation of Ts cell activity.

Accessory Cells Determined the MHC Restriction of In Vitro Activated Ts Cells. The activation and effector function of a number of T cell populations has previously been shown to involve MHC-restricted cellular interaction events. Since it was established in the experiments presented above that in vitro Ts cell activation required the participation of accessory cells as well as T cells, it was of interest to determine whether these interactions between T cells and accessory cells could determine MHC restrictions in the activity of the Ts cells generated. Experiments were therefore
Table I

Accessory Cells Are Required for Induction of Ts Cells In Vitro

| Accessory cells added | Antigen stimulation in vitro | Ts cell treatment$|$ Anti-TNP IgG PFC/culture$| |
|----------------------|-----------------------------|-----------------------------|
| $\ldots$ $\ldots$ | $\ldots$ | 1,987 $\pm$ 240 |
| $-$ | KLH | Untreated | 1,776 $\pm$ 93 |
| $+$ | KLH | Untreated | 1,948 $\pm$ 189 |
| $+$ | FGG | Untreated | 1,819 $\pm$ 261 |
| $+$ | KLH | C treated | 748 $\pm$ 37 |
| $+$ | KLH | Anti-Thy-1.2 + C | 849 $\pm$ 38 |
| $+$ | KLH | Anti-Thy-1.2 + C | 2,088 $\pm$ 101 |

$^*$ KLH-primed B10 T cells were passed over Sephadex G-10 columns and were cultured for 3 d in the presence or absence of additional unprimed B10 accessory cells and of 100 $\mu$g/ml of the indicated antigen.

$|$ After preculture, cells were either untreated, treated with C alone, or treated with anti-Thy-1.2 + C.

$|$ $3 \times 10^6$ TNP-KLH-primed B10 spleen cells were stimulated in vitro with 0.001 $\mu$g/ml TNP-KLH in the presence or absence of $1 \times 10^6$ treated or untreated Ts cells.

$|$ Ts cells were not added to the responding cultures.

These results demonstrated that antigen-primed (A × B)$F_1$ T cell populations generate Ts cells as a result of preculturing with parent$A$ accessory cells and antigen, and that the $F_1$ Ts cells activated under these conditions are MHC restricted in their ability to suppress the responses of parent$A$ but not parent$B$ (B + accessory) cells. In addition, the failure of parent$A$-restricted or parent$B$-restricted Ts
Accessory Cells Determine the MHC Restriction of Ts Cell Function

| Group | Accessory cells added | Antigen stimulation in vitro | Anti-TNP IgG PFC/culture |
|-------|-----------------------|-----------------------------|--------------------------|
|       |                       | B10                         | B10.A                    | (B10 × B10.A)F1          |
| A     | ...§                  | 1,030 ± 163                 | 810 ± 58                 | 1,276 ± 375              |
| B     | −                     | 1,020 ± 45                  | 942 ± 116                | ND                       |
| C     | −                     | 885 ± 76                    | 630 ± 52                 | 1,074 ± 19               |
| D     | B10                   | 1,132 ± 123                 | 836 ± 117                | ND                       |
| E     | B10                   | 40 ± 9                      | 630 ± 29                 | 1,175 ± 111              |
| F     | B10.A                 | 1,155 ± 109                 | 962 ± 34                 | ND                       |
| G     | B10.A                 | 413 ± 29                    | 0                        | 1,013 ± 105              |
| H     | [Group E + group G]§ | ND                          | ND                       | 0                        |

*KLH-primed (B10 × B10.A)F1 T cells were passed over Sephadex G-10 columns and were precultured in the presence or absence of additional unprimed accessory cells and in the presence or absence of 100 μg/ml KLH.

§ 3 × 10⁶ TNP-primed (B + accessory) cells from the indicated strains were co-cultured with 1 × 10⁶ KLH-primed (B10 × B10.A)F1 Th cells. Cultures were stimulated in vitro with 0.001 μg/ml TNP-KLH in the presence or absence of 1 × 10⁶ Ts cells.

§ Ts cells were not added to the cultures.

§§ Mixture of 1 × 10⁶ group E Ts and 1 × 10⁶ group G Ts.

Both Antigen-specific and Antigen-nonspecific MHC-restricted Ts Cells Are Activated in (A × B)F1 T Cells by Stimulation with Parental Accessory Cells. It has been demonstrated (17) that two distinct Ts populations are induced by preculturing antigen-primed T cells in the presence of specific antigen: Lyt-1⁺2⁻ Ts cells that mediate antigen-nonspecific suppression and Lyt-1⁻2⁺ Ts cells that mediate antigen-specific suppression. It was therefore of interest to evaluate whether both of these Ts populations were generated under the conditions of the present study and whether both of these Ts populations were thus MHC restricted in their effector functions. KLH-primed (B10 × B10.A)F1 T cells were depleted of accessory cells and were precultured with either B10 or B10.A accessory cells and with 100 μg/ml KLH. After 3 d of preculture, Ts cells were treated with either anti-Lyt-1.2 + C, anti-Lyt-2.2 + C, or anti-Lyt-1.2 + anti-Lyt-2.2 + C, and the residual cells were tested for their suppressive activities on responses of either KLH- or FGG-primed (B10 × B10.A)F1 Th cells and TNP-primed B10 or B10.A (B + accessory) cells to TNP-KLH or TNP-FGG, respectively (Table III). Ts cells activated in the presence of B10 accessory cells suppressed the responses of B10 but not B10.A (B + accessory) cells to TNP-KLH and TNP-FGG. Treatment of activated Ts cells with anti-Lyt-1.2 + C eliminated the antigen-nonspecific Ts cell activity that was detected on TNP-FGG responses but did not affect the antigen-specific Ts cell activity measured on TNP-KLH responses, confirming previous results (17). On the other hand, treatment of Ts cells with anti-Lyt-2.2
Table III

Antigen-specific and Antigen-nonspecific Ts Cells Are MHC-restricted in their Function

| Accessory cells added | Ts cell treatment§ | Anti-TNP IgG PFC/culture* |
|-----------------------|--------------------|---------------------------|
|                       |                    | B10 | B10.A |
|                       | TNP-KLH            | TNP-FGG | TNP-KLH | TNP-FGG |
| ...                   | 1,460 ± 197        | 1,082 ± 42 | 1,276 ± 106 | 985 ± 74 |
| - Untreated           | 1,170 ± 23         | 924 ± 160 | 1,302 ± 63  | 897 ± 84 |
| B10 Untreated         | 78 ± 26            | 8 ± 8     | 1,000 ± 74  | 1,082 ± 133 |
| B10 Anti-Lyt-1.2 + C  | 176 ± 91           | 765 ± 21  | 1,295 ± 144 | 985 ± 162 |
| B10 Anti-Lyt-2.2 + C  | 61 ± 61            | 35 ± 35   | 1,302 ± 101 | 1,179 ± 91 |
| B10 Anti-Lyt-1.2 + anti-Lyt-2.2 + C | 1,416 ± 71 | 1,003 ± 139 | 1,425 ± 64 | 1,047 ± 63 |
| B10.A Untreated       | 1,328 ± 101        | 1,056 ± 52 | 211 ± 121 | 17 ± 8 |

* 3 × 10⁶ TNP-primed, (RAMB + C)-treated (B + accessory) cells from the indicated strain were co-cultured with 1 × 10⁶ KLH-primed or FGG-primed (B10 × B10.A)F₁ Th cells. Cultures were stimulated with 0.001 µg/ml of TNP-KLH or TNP-FGG, respectively.
§ KLH-primed (B10 × B10.A)F₁ T cells were passed over Sephadex G-10 columns and were cultured for 3 d with 100 µg/ml KLH in the presence or absence of added irradiated unprimed accessory cells.
§ Ts cells were treated with the indicated antiserum + C after preculture. 1 × 10⁶ residual cells were added to the cultures.
|| Ts cells were not added to the cultures.

+ C failed to eliminate Ts cell activity on either TNP-KLH or TNP-FGG (Table III). This failure to eliminate suppression was likely the result of remaining Lyt-1⁻²⁺ antigen-nonspecific Ts cells (17). In fact, combined treatment of Ts cells with anti-Lyt-1⁻2⁺ anti-Lyt-2.2 + C completely eliminated Ts activity (Table III), confirming this interpretation. These results demonstrated that antigen-primed (A × B)F₁ T cells precultured with parentA accessory cells in the presence of specific antigen are capable of generating both antigen-specific Lyt-1⁻²⁺ Ts cells and antigen-nonspecific Lyt-1⁻²⁺ Ts cells and that both of these Ts cell populations are MHC restricted in their effector functions.

**T Cell Recognition of I-A-encoded Determinants on Accessory Cells Is Required for the Activation of I-A-restricted Ts Cells.** It was next determined what region(s) or subregion(s) of the MHC encodes the determinants that restrict the induction and effector function of the Ts cells characterized above. KLH-primed (A.TH × A.TL)F₁ T cells were depleted of accessory cells and precultured with either A.TH, A.TL, or (A.TH × A.TL)F₁ accessory cells in the presence of 100 µg/ml KLH. Precultured cells were then tested for their suppressive activities on the responses of KLH-primed (A.TH × A.TL)F₁ Th cells and TNP-primed A.TH, A.TL, or (A.TH × A.TL)F₁ (B + accessory) cells to TNP-KLH (Table IV). (A.TH × A.TL)F₁ Ts cells activated by A.TH accessory cells suppressed the responses of A.TH (B + accessory) cells but failed to suppress the responses of A.TL and (A.TH × A.TL)F₁ (B + accessory) cells. In contrast, (A.TH × A.TL)F₁ Ts cells activated by A.TL accessory cells suppressed the responses of A.TL but not A.TH or (A.TH × A.TL)F₁ (B + accessory) cells (Table IV). These results demonstrate that I region-encoded determinants expressed on the accessory cells mediating Ts cell activation determine the I region-restricted effector activity of these Ts cells.

To further evaluate which I subregion(s) is involved in restricting the activation
TABLE IV

Recognition of I Region Products Is Required for Ts Cell Function

| Accessory cells added‡ | Origin of H-2 | Anti-TNP IgG PFC/culture* |
|------------------------|--------------|--------------------------|
|                        | K | I | D | A.TH | A.TL |
|                        |   |  |  |      |      |
| ...§                   |   |  |  |      |      |
| A.TH                   | s | s | d | 52 ± 30 | 932 ± 106 |
| A.TL                   | s | k | d | 642 ± 76 | 88 ± 46 |
| (A.TH × A.TL)F1       | s/s | s/k | d/d | 132 ± 60 | 114 ± 35 |

* 3 × 10⁶ TNP-primed, (RAMB + C)-treated (B + accessory) cells from the indicated strain were co-cultured with 1 × 10⁶ KLH-primed (A.TH × A.TL)F1 Th cells. Cultures were stimulated with 0.001 μg/ml TNP-KLH.
‡ KLH-primed (A.TH × A.TL)F1 T cells were passed over Sephadex G-10 columns and were cultured for 3 d with 100 μg/ml KLH in the presence or absence of added irradiated unprimed accessory cells. 1 × 10⁶ activated Ts cells were added to the cultures.
§ Ts cells were not added to the cultures.

TABLE V

Recognition of K and/or I-A Products Is Required for Ts Function

| Accessory cells added‡ | Origin of H-2 | Anti-TNP IgG PFC/culture* |
|------------------------|--------------|--------------------------|
|                        | K | A | B | J | E | C | S | D | B10 | B10.A | B10.A(4R) |
|                        |   |   |   |   |   |   |   |   | B10 |      |          |
| ...§                   |   |   |   |   |   |   |   |   | 2,020 ± 257 | 1,905 ± 191 | 1,411 ± 108 |
| B10                    | b | b | b | b | b | b | b | b | 2,174 ± 117 | 1,833 ± 69 | 1,689 ± 150 |
| B10.A(3R)              | b | b | b | b | k | d | d | d | 796 ± 26 | 2,083 ± 194 | 1,920 ± 106 |
| B10.A(5R)              | b | b | b | k | k | d | d | d | 432 ± 33 | 1,574 ± 125 | 1,449 ± 101 |
| B10.A(4R)              | k | k | b | k | b | b | b | b | 1,764 ± 146 | 403 ± 11 | 681 ± 210 |
| B10.A                  | k | k | k | k | k | d | d | d | 2,030 ± 180 | 871 ± 11 | 643 ± 135 |

* 3 × 10⁶ TNP-primed, (RAMB + C)-treated (B + accessory) cells from the indicated strain were co-cultured with 1 × 10⁶ KLH-primed (B10 × B10.A)F1 Th cells. Cultures were stimulated with 0.001 μg/ml TNP-KLH.
‡ KLH-primed (B10 × B10.A)F1 T cells were passed over Sephadex G-10 columns and were cultured for 3 d with 100 μg/ml KLH in the presence or absence of added irradiated unprimed spleen cells. 1 × 10⁶ activated Ts cells were added to the cultures.
§ Ts cells were not added to the cultures.

and function of MHC-restricted Ts cells, KLH-primed (B10 × B10.A)F1 T cells were depleted of accessory cells and precultured with B10, B10.A(3R), B10.A(4R), B10.A(5R), or B10.A accessory cells in the presence of 100 μg/ml KLH. Induced Ts cells were then tested for their suppressive activities on the responses of KLH-primed (B10 × B10.A)F1 Th cells and TNP-primed B10, B10.A, or B10.A(4R) (B + accessory) cells to TNP-KLH (Table V). B10 (B + accessory) cell responses were effectively suppressed by F1 Ts cells induced in the presence of either B10, B10.A(3R), or B10.A(5R) accessory cells. On the other hand, B10.A and B10.A(4R) (B + accessory) cell responses were suppressed by Ts cells induced in the presence of either B10.A or B10.A(4R) accessory cells. Other combinations failed to permit efficient suppression (Table V). These results suggested that K and/or I-A region homology between the accessory cells used for Ts cell activation and the (B + accessory) cells in the responding populations is required for MHC-restricted Ts cell-mediated suppression.
Taken together, the above results suggest that I-A homology between the accessory cells functioning in Ts activation and the (B + accessory) cells in responding populations is required for Ts cell activation and function.

The possibility that Ts cell activation requires recognition of I-A-encoded determinants on accessory cells was further evaluated in studies investigating the effect of anti-Ia antibody on Ts cell generation. KLH-primed (B10 × B10.BR)F1 T cells (not depleted of F1 accessory cells) were precultured in the presence of 100 µg/ml KLH, and in the presence of titrated amounts of monoclonal anti-I-A<sup>k</sup> antibody, and the generation of Ts cells evaluated (Fig. 1). In the absence of anti-I-A<sup>k</sup> antibody, activated Ts cells suppressed equally well the responses of B10 and B10.BR (B + accessory) cells (Fig. 1). The addition of anti-I-A<sup>k</sup> antibody during preculture blocked the generation of B10.BR (I-A<sup>k</sup>)-restricted Ts cells. In striking contrast, anti-I-A<sup>k</sup> antibody did not interfere with the generation of B10 (I-A<sup>b</sup>)-restricted Ts cells in the same cultures (Fig. 1). Since both the T cells and the accessory cells present in these precultures were uniformly (B10 × B10.BR)F1 in phenotype, the inhibitory effect of anti-I-A<sup>k</sup> antibody observed here was not the nonspecific consequence of antibody binding to either T cells or accessory cells, but was rather a selective effect on the activation of an MHC-restricted F1 Ts cell population. These results suggest that T cell recognition of I-A-encoded Ia determinants expressed on accessory cells is required for Ts cell activation.

The MHC-restricted Repertoire of Ts Cells is influenced by their Maturation Environment. The preceding results suggested that T cell recognition of I-A molecules on accessory cells is required for Ts cell activation and function. Because it has been demonstrated (26–31) that the T cell maturation environment can profoundly influence the repertoire of certain T cells for recognition of "self" I-A products on accessory cells and/or B cells, it was of interest to determine whether the T cell maturation environment can influence the Ts cell repertoire as well. (B10 × B10.BR)F1 → B10

![Fig. 1. Anti-I-A<sup>k</sup> antibody inhibits the activation of I-A<sup>k</sup>-restricted Ts cells. KLH-primed (B10 × B10.BR)F1 T cells were precultured in the absence (Δ) or presence (○, ▽, △) of 100 µg/ml KLH, and in the absence (Δ, ○) or presence of 5% (●, 10% (▲), or 20% (●) anti-I-A<sup>k</sup> antibody. The suppressive activities of the resulting populations were tested on the responses of 1 × 10<sup>6</sup> KLH-primed (B10 × B10.BR)F1 Th cells and 3 × 10<sup>5</sup> TNP-primed, (RAMB + C)-treated B10 (A) or B10.BR (B) (B + accessory) cells.](image-url)
mice were primed in vivo with KLH and stimulated in vitro with KLH in the presence of (B10 × B10.BR)F1 accessory cells. The induced Ts cells were tested for their suppressive activities on the responses of KLH-primed B6C3F1 Th cells and TNP-primed B10 or B10.BR (B + accessory) cells (Table VI). Ts cells induced in KLH-primed B6C3F1 T cells by stimulation with KLH suppressed the responses of B10 as well as B10.BR (B + accessory) cells (Table VI). On the other hand, Ts cells induced in KLH-primed (B10 × B10.BR)F1 → B10 T cells by stimulation with (B10 × B10.BR)F1 accessory cells and KLH, suppressed the responses of B10 but not B10.BR (B + accessory) cells (Table VI), in spite of the fact that these Ts cells were genotypically (B10 × B10.BR)F1 cells and that (B10 × B10.BR)F1 accessory cells were present during in vitro stimulation of Ts cells. These results demonstrate that (A × B)F1 T cells that had matured in a parentA environment were able to generate Ts cells specific for parentA (B + accessory) cell responses but failed to generate Ts cells specific for parentB (B + accessory) cell responses. Thus, the T cell maturation environment can determine the self MHC-restricted repertoire of Ts cells in a fashion similar to that previously described for other functional T cell populations.

Neither MHC Homology Nor Recognition of MHC Determinants Between Th Cells and Ts Cells Is Required for Ts Cell Function. It has been reported in several systems (1–11) that MHC restrictions exist in the activation and/or function of Ts cells. The results presented above demonstrated that recognition of MHC determinants on (F1) Th cells is not sufficient to allow suppression in the present studies, but that Ts cell activity is observed only when responding (B + accessory) cells express the appropriate MHC determinants. Nevertheless, these results did not exclude the possibility that MHC restriction also exists in the interaction of Th cells and Ts cells. Additional experiments were therefore designed to address this question. Ts cells were induced in B10 → (B10 × B10.A)F1 T cells or B10.A → (B10 × B10.A)F1 T cells by in vivo priming with KLH-pulsed (B10 × B10.A)F1 accessory cells and in vitro stimulation with KLH in the presence of either B10 or B10.A accessory cells. In addition, Ts cells were induced in KLH-primed (B10 × B10.A)F1 → B10.A T cells by stimulation with KLH and

### Table VI

| T cell source | Antigen stimulation in vitro | Anti-TNP IgG PFC/culture |
|---------------|-----------------------------|--------------------------|
| B6C3F1        | −                           | 1,276 ± 47, 1,060 ± 40   |
| B6C3F1        | +                           | 484 ± 148, 201 ± 173     |
| (B10 × B10.BR)F1 → B10 | − | 1,982 ± 95, 1,152 ± 92 |
| (B10 × B10.BR)F1 → B10 | + | 422 ± 65, 1,022 ± 129 |

*KLH-primed T cells of indicated origin were cultured for 3 d with unprimed (B10 × B10.BR)F1 accessory cells in the presence or absence of 100 μg/ml KLH.

† 3 × 10⁴ TNP-primed, (RAMB + C)-treated B10 or B10.BR (B + accessory) cells were co-cultured with 1 × 10⁶ KLH-primed B6C3F1 Th cells. Cultures were stimulated with 0.001 μg/ml TNP-KLH in the presence or absence of 1 × 10⁶ Ts cells.

§ Ts cells were not added to the cultures.
LA RESTRICTED T SUPPRESSOR CELLS

(B10 × B10.A)F1 accessory cells. These induced Ts cells were then tested for their suppressive activities on the responses of TNP-primed B10 or B10.A (B + accessory) cells and Th cells from B10 → (B10 × B10.A)F1 or B10.A → (B10 × B10.A)F1 mice primed in vivo with KLH-pulsed (B10 × B10.A)F1 accessory cells (Table VII). Ts cells induced in B10 → (B10 × B10.A)F1 T cells by stimulation with B10 accessory cells and KLH suppressed the responses of B10 but not B10.A (B + accessory) cells, regardless of the genotype of the Th cells functioning in these responses (Table VII). In contrast, Ts cells induced in B10 → (B10 × B10.A)F1 T cells by stimulation with B10.A accessory cells and KLH suppressed the responses of B10.A but not B10 (B + accessory) cells (Table VII). Moreover, Ts cells induced in B10.A → (B10 × B10.A)F1 T cells by stimulation with B10 or B10.A accessory cells, showed the same pattern (Table VII). In addition, Ts cells induced in (B10 × B10.A)F1 → B10.A T cells suppressed the responses of B10.A (B + accessory) cells and Th cells of either B10 → (B10 × B10.A)F1 or B10.A → (B10 × B10.A)F1 origin, but failed to suppress the responses of B10 (B + accessory) cells and the same Th cell populations (Table VII). These results demonstrated that (a) no requirement exists for MHC homology between Ts cells and Th cells, and (b) no requirement can be identified for Ts cell recognition of MHC determinants on Th cells.

Next, it was determined whether Th cells have to recognize MHC determinants on Ts cells. Ts cells were induced in either B10 → (B10 × B10.A)F1 or B10.A → (B10 × B10.A)F1 T cells, which were primed in vivo with KLH-pulsed (B10 × B10.A)F1 accessory cells, by stimulation with either B10 or B10.A accessory cells and KLH and were tested for their suppressive activities on the responses of TNP-primed B10 or

### Table VII

**Ts Cell Recognition of MHC Determinants on Th Cells Is Not Required for Ts Cell Function**

| T cells from: | Accessory cells added | Antigen stimulation in vitro | Anti-TNP IgG PFC/culture‡ | B10 (B + accessory) cells | B10.A (B + accessory) cells |
|--------------|-----------------------|-----------------------------|---------------------------|--------------------------|---------------------------|
| ... §        | ...                   | ...                         |                           | B10 → F1 Th              | B10.A → F1 Th             |
| B10 → F1∥   | −                     | +                           | 1,918 ± 140               | 1,584 ± 169              | 1,161 ± 132               | 1,355 ± 230               |
| B10 → F1    | B10                   | +                           | 2,182 ± 98                | 1,645 ± 132              | 994 ± 68                  | 1,434 ± 298               |
| B10 → F1    | B10.A                 | +                           | 624 ± 68                  | 567 ± 53                | 897 ± 52                  | 1,095 ± 10                |
| B10.A → F1  | −                     | +                           | 2,201 ± 249               | 2,032 ± 184              | 79 ± 45                   | 150 ± 80                  |
| B10.A → F1  | B10                   | +                           | 1,273 ± 89                | 1,865 ± 217              | 906 ± 91                  | 1,042 ± 32                |
| B10.A → F1  | B10.A                 | +                           | 123 ± 23                  | 88 ± 17                 | 765 ± 80                  | 1,066 ± 103               |
| B10.A → F1  | B10.A                 | +                           | 1,645 ± 23                | 1,390 ± 97              | 17 ± 17                   | 290 ± 54                  |
| F1 → B10.A  | F1                    | −                           | 2,657 ± 399               | 1,988 ± 163              | 1,232 ± 89                | 1,654 ± 162               |
| F1 → B10.A  | F1                    | +                           | 2,182 ± 131               | 1,522 ± 132              | 281 ± 68                  | 457 ± 76                  |

* KLH-primed T cells of indicated origin were passed over Sephadex G-10 columns and were cultured for 3 d in the presence or absence of additional unprimed accessory cells of the indicated strain and of 100 μg/ml KLH.

‡ 3 × 10⁶ TNP-primed B10 or B10.A (B + accessory) cells were co-cultured with 1 × 10⁶ KLH-primed Th cells of the indicated strain. Cultures were stimulated with 0.001 μg/ml TNP-KLH in the presence or absence of 1 × 10⁶ Ts cells.

§ Ts cells were not added to the cultures.

∥ (B10 × B10.A)F1.
B10.A (B + accessory) cells and KLH-primed (B10 × B10.A)F1 → B10.A Th cells to 0.001 μg/ml TNP-KLH (Table VIII). (B10 × B10.A)F1 → B10.A Th cells were restricted to cooperating with B10.A but not B10 (B + accessory) cells, confirming previous results (19). The responses generated by B10.A (B + accessory) cells and (B10 × B10.A)F1 → B10.A Th cells were suppressed by Ts cells induced in either B10 → (B10 × B10.A)F1 or B10.A → (B10 × B10.A)F1 T cells by stimulation with B10.A but not B10 accessory cells (Table VIII). These results suggest that Th cell recognition of Ts cell MHC determinants is not required for Ts cell effector function. Taken together, the results described above suggest that neither MHC homology nor recognition of MHC determinants is required for any interactions between Th cells and Ts cells.

The preceding results demonstrated that two distinct Ts populations are generated by the preculture of antigen-primed T cells with corresponding antigen and that both of these Ts cells were MHC-restricted in their function. Although it was demonstrated above that at least one of these Ts pathways required neither MHC homology nor recognition of MHC determinants between Th cells and Ts cells, it remained possible that one of these Ts cell populations did require MHC-restricted interactions between Th cells and Ts cells. Further studies were therefore carried out in which anti-Lyt treatment was used to independently evaluate the antigen-nonspecific Lyt-1−2− and the antigen-specific Lyt-1−2+ Ts pathways. In experiments similar to those presented above, it was found that neither of these Ts pathways expresses any requirement for MHC-restricted interaction between Ts and Th cells (data not shown).

Ts Cell Function Is Intimately Related to the MHC-restricted Interaction Between Th Cells and B Cells and/or Accessory Cells. The results presented above failed to demonstrate any MHC restriction in the interaction between Th cells and Ts cells. They also

### Table VIII

| Ts cell induction* | Anti-TNP IgG PFC/culture‡ |
|-------------------|---------------------------|
| T cells from:     | Accessory cells added     | B10.A | B10  |
| ⋯⋯§               | ⋯⋯                        | 1,716 ± 124 | 149 ± 112 |
| B10 → F₁           | B10.A                     | 1,592 ± 83  | ND\| |
| B10 → F₁           | B10.A                     | 1,406 ± 83  | ND  |
| B10.A → F₁         | B10.A                     | 193 ± 71    | ND  |
| B10.A → F₁         | B10.A                     | 1,513 ± 96  | ND  |
| B10.A → F₁         | B10.A                     | 1,320 ± 205 | ND  |
| B10.A → F₁         | B10.A                     | 17 ± 17     | ND  |

* KLH-primed T cells of indicated origin were passed over Sephadex G-10 columns and were cultured with 100 μg/ml KLH for 3 d in the presence or absence of additional unprimed accessory cells.
‡ 3 × 10⁶ TNP-primed, (RAMB + C)-treated B10 or B10.A (B + accessory) cells were co-cultured with KLH-primed (B10 × B10.A)F1 → B10.A Th cells. Cultures were stimulated with 0.001 μg/ml TNP-KLH in the presence or absence of 1 × 10⁶ Ts cells.
§ Ts cells were not added to the cultures.
|| (B10 × B10.A)F1.
¶ Not determined.
indicated that Ts cell recognition of the appropriate MHC products on (A × B)F₁ (B + accessory) cells was itself not sufficient to allow suppression of B cell responses. These findings were thus consistent with the possibility that these Ts cells did not function to directly suppress the functions of Th cells, B cells, or accessory cells per se, but rather that they might interfere with the MHC-restricted interaction previously shown to occur between Th cells and B cells and/or accessory cells (19, 20). To directly assess this possibility, the following experiments were carried out. T cells from B10.A → B6AF₁ mice primed in vivo with KLH-primed B6AF₁ accessory cells were depleted of accessory cells and then preincubated with KLH and B10 or B10.A accessory cells. Suppressive activity was then tested on the responses of KLH-primed B6AF₁ → B10 or B10 → B6AF₁ Th cells and TNP-primed (B10 × B10.A)F₁ (B + accessory) cells (Table IX). B6AF₁ → B10 Th cells generated responses with B10 but not B10.A (B + accessory) cells (data not shown), indicating that these Th cells were B10 restricted in their helper function, whereas B10 → B6AF₁ Th cells cooperated efficiently with either B10 or B10.A (B + accessory) cells (Table IX). When B10-restricted B6AF₁ → B10 Th cells cooperated with (B10 × B10.A)F₁ (B + accessory) cells, responses were generated that were strongly suppressed by B10.A → B6AF₁ Ts cells activated by B10 but not B10.A accessory cells (Table IX). Thus, when chimeric (H-2b × H-2b)F₁ → H-2b Th cells were restricted to recognizing H-2b but not H-2b determinants on F₁ (B + accessory) cells, H-2b- but not H-2b-restricted Ts cells were able to suppress antibody responses. These results demonstrate that the effect of Ts cells is not a direct inhibitory effect on B cells, accessory cells, or Th cells expressing a given MHC phenotype, but rather is a specific effect on the interaction of MHC-restricted Th cells with responding B cells and/or accessory cells.

Discussion

To evaluate the cell/cell interactions involved in the Ts cell regulation of T-dependent, MHC-restricted B cell activation, the role of genetically restricted recog-
nition events in the generation and function of Ts cells was studied in the present report. First, it was demonstrated that the presence of accessory cells as well as antigen-primed T cells was required for the antigen-specific in vitro activation of both the Lyt-2-2+ Ts cells mediating antigen-specific suppression and Lyt-1-2+ Ts cells mediating antigen-nonspecific suppression. Furthermore, it was demonstrated that both of these Ts cell populations were MHC-restricted both at the level of their in vitro activation and at the level of their effector function. In antigen-primed (A × B)F1 T cell populations, parentA-restricted Ts cells were generated by stimulation with antigen in the presence of parentA but not parentB accessory cells, while parentB-restricted Ts cells were similarly generated only in the presence of parentB accessory cells. The MHC restriction of these Ts cells was expressed at the level of their effector function, such that parentA-restricted (A × B)F1 Ts cells suppressed the T cellDependent responses generated by (A × B)F1 Th cells in cooperation with parentA (B + accessory) cells but did not suppress the responses generated by the same F1 Th cells in cooperation with parentB (B + accessory) cells. The use of intra-H-2 recombinant strains demonstrated that these restrictions involved a requirement for recognition of products encoded in the I-A subregion. Several conclusions can be drawn concerning the nature of the MHC restriction that was observed at the level of Ts cell activation. First, it should be noted that this restriction does not represent a requirement for I-A homology, but is rather a requirement for active Ts cell recognition of determinants expressed on accessory cells. Thus, (A × B)F1 Ts cells that had been activated by parentA accessory cells suppressed only the response of parenth (B + accessory) cells, although these F1 Ts cells were equally homologous with both parentA and parentB (B + accessory) cells. In addition, antigen-primed (A × B)F1 → parentA chimeric T cells failed to generate parentB-specific Ts cells even when stimulated with (A × B)F1 accessory cells and antigen, suggesting that these chimeric T cells lack the parentB specific Ts cell repertoire. In contrast, parentA → (A × B)F1 T cells primed in vivo with antigen-pulsed (A × B)F1 accessory cells were able to generate parentA- or parentB-specific Ts cells upon stimulation with antigen and either parentA or parentB accessory cells, respectively, demonstrating that these chimeric T cells (themselves of parentA origin) have both parentA- and parentB-specific Ts cell repertoires that can be activated by MHC-restricted interactions with the appropriate accessory cells. Collectively, these findings demonstrate that the MHC-restricted Ts cell repertoire, like those of other functional T cell populations, is strongly influenced by the T cell maturation environment, consistent with the recent report by Flood et al. (32). The MHC restriction that functions during the in vitro activation of antigen-specific and antigen-nonspecific Ts cells shows specificity for both antigen and accessory cell "self" I-A products, and the ability to selectively block the generation of I-Ak-restricted Ts cells with a monoclonal anti-I-Ak antibody further suggests that the restricting element recognized by Ts precursors is in fact the serologically defined Ia product of this subregion.

It has been reported by Feldmann and Kontiainen (33) that accessory cells either were not required for Ts cell induction or were required in numbers considerably lower than required for Th cell induction in vitro. Pierres and Germain (34) similarly concluded that in vitro Ts cell generation was favored by a reduction in accessory cell number. While not in direct conflict with the data presented by these authors, the present studies have directly demonstrated that accessory cells are required for the in
vitro activation of at least certain Ts cells and that the MHC determinants expressed by these accessory cells determine the MHC-restriction specificity of the Ts cells activated. A number of laboratories have identified requirements for recognition of MHC products in Ts cell function (1-12, 32). The results presented in this report demonstrated that recognition of I-A-encoded determinants is required for both the induction and function of the Ts cells studied here. There was, in contrast, no apparent restriction for recognition of the products of other I subregions. These findings are in particular contrast with the results of a large number of studies in which it was found that I-J-encoded determinants were critical restricting elements in the sequential cell/cell interactions involved in Ts cell pathways (1-11, 32). The present study failed to demonstrate any requirement for either homology of I-J-encoded determinants or Ts cell recognition of I-J-encoded determinants on Th cells. It has not been determined whether the Ts cells described in this report express cell surface I-J-encoded determinants.

The restrictions that were observed in the present studies suggest that there exists a mechanism of T cell-mediated regulation which is quite distinct from those previously proposed. Whereas the in vitro activation of the Ts cells mediating both antigen-specific and antigen-nonspecific suppression demonstrated specificity for antigen in association with the appropriate I-A-encoded determinants on accessory cells, the observation that neither parentA-restricted nor parentB-restricted Ts cells were able to suppress the responses of (A × B)F₁ Th cells and (A × B)F₁ (B + accessory) cells implied that the recognition of appropriate I-A products on B cells and/or accessory cells is not sufficient to permit suppression. Indeed, experiments using parentA-restricted Th cells from (A × B)F₁ → parentA chimeras demonstrated that parentA-restricted Ts cells, but not parentB-restricted Ts cells could suppress responses mediated by (A × B)F₁ (B + accessory) cells and (A × B)F₁ → parentA Th cells. Thus, when (A × B)F₁ Th cells were restricted to recognizing only parentA MHC determinants on responding (A × B)F₁ (B + accessory) cells, these responses were suppressed by parentA-restricted Ts cells. These results demonstrate that the I-A-restricted function of both antigen-specific and antigen-nonspecific Ts cells is related not to recognition of genotypic B cell and/or accessory cell I-A products alone, but rather that Ts function is intimately related to the MHC-restricted interaction between Th cells and responding (B + accessory) cells.

Since the antigen-specific Ts cells characterized in this report are specific in their activation requirements for antigen plus I-A product, the most straightforward interpretation of these findings is that the effector function of these cells also requires recognition of antigen plus I-A product. As noted above, the target of this I-A-restricted recognition does not appear to be the codominantly expressed genotypically determined I-A product on T cells, B cells, or accessory cells. In contrast, the results presented here are consistent with a mechanism in which Ts cells recognize both the specific antigen and the appropriate I-A product bound to the appropriate clonally expressed Th cell receptor(s). This recognition event would then lead to the functional inactivation of only those Th cells expressing specificity for the appropriate antigen plus I-A. Since it has been shown that the same Th cells can activate B cells through both MHC-restricted and unrestricted pathways (20), the finding that the Ts cells described here selectively affect the MHC-restricted Th cell activation of Lyb-5⁺ B cells but not the MHC-unrestricted activation of Lyb-5⁺ B cells suggests that any Ts
cell effects on Th cells must selectively interfere with only certain Th cell functions. This model of Ts cell function has several implications. If Ts cells in fact recognize antigen plus I-A products as "presented" by the Th cell receptor, it might be predicted that the Ts cell acting upon a given Th cell would of necessity recognize epitopes on antigen and/or I-A products which are different from those recognized by that Th cell. That is, in the absence of multiple (polymeric) cross-reactive determinants on the same antigen and/or I-A molecule, steric constraints would be expected to prevent both Th and Ts cell receptors from simultaneously recognizing the same determinant. The existence of distinct "suppressor determinants" and "helper determinants" on a single antigen molecule has in fact been proposed for the response to poly-(Glu, Ala, Tyr) by Schwartz et al. (35) and for the response to lysozyme by Adorini et al. (36); and the potential importance of steric constraints for the interaction of Th cells and B cells has been discussed by Berzofsky (37). To further assess this issue, both MHC-restricted cloned Ts cells and MHC-restricted cloned Th cells have recently been generated, and studies of their interactions and fine specificities are now in progress.

Alternative mechanisms consistent with the observed restrictions in Ts cell activity involve Ts cell recognition of determinants on B cells. In one such mechanism, Ts cells or Ts factors might recognize and bind to antigen and I-A product on B cells, thus directly competing with Th cells of the same specificity. It is also possible that Ts cells recognize determinants presented jointly by the interacting Th cell and B cell. Ts cells could, for example, recognize antigen presented by the Th cell receptor and the B cell I-A product being presented to the same Th cell. This alternative, although subject to significant steric constraints, would also account for the selective effect of Ts cells on the MHC-restricted pathway of B cell activation.

Finally, while the experiments presented in this report failed to identify any genotype-related MHC restriction in the interactions between Th cells and Ts cells, these data do not exclude the possibility that Ts cells directly recognize non-MHC determinants on Th cells. Therefore, one interpretation of the findings presented above is that Ts cells directly interact with the I-A-restricted receptors expressed by Th cells and in that manner inhibit the MHC-restricted Th cell recognition of B cells and/or accessory cells required for response. This possibility is currently being evaluated by assessing the presence of any non-MHC, e.g., IgH-linked restrictions in the interaction of Ts and Th cells. It will also be of interest to study the specificity of cloned Ts cells directly, since it would be unlikely that an antireceptor specificity is expressed by the same cloned Ts cell which also recognizes antigen plus Ia.

The studies presented here have characterized two Ts cell populations that appear to function through a unique and highly specific mechanism. The activation of these Ts cells requires active recognition by T cells of accessory cell I-A products, and is mediated by an MHC-restricted Ts cell repertoire which is profoundly influenced by the T cell maturation environment. The mode of action of these Ts cells appears to involve a specific effect upon the MHC-restricted interaction between Th cells and B cells and/or accessory cells that is necessary for the generation of IgG responses by Lyb-5+ B cells. Further analysis of the mechanism of this T cell-mediated suppression is now being carried out in studies using monoclonal Ts cell populations and their soluble products.

Summary

The present studies were carried out to characterize the cellular interactions involved in the activation and function of the antigen-specific and antigen-nonspecific
T suppressor (Ts) cells that regulate the IgG responses of Lyb-5- B cells. The in vitro activation of both Lyt-1+2- antigen-nonspecific Ts cells and Lyt-1-2+ antigen-specific Ts cells was shown to require the interaction of accessory cells and antigen-primed T cells. It was further demonstrated that this interaction was major histocompatibility complex (MHC)-restricted in that T cell recognition of I-A-encoded determinants on accessory cells was required for Ts cell activation.

The activation of antigen-primed (A × B)F1 T cells with antigen in the presence of parentA or parentB accessory cells resulted, respectively, in the generation of parentA-restricted or parentB-restricted Ts cells. ParentA-restricted F1 Ts cells suppressed the responses generated by (A × B)F1 T helper (Th) cells cooperating with parentA (B + accessory) cells but did not suppress responses by the same (A × B)F1 Th cell population cooperating with parentB (B + accessory) cells. Neither parentA-restricted Ts cells alone nor parentB-restricted Ts cells alone suppressed the responses of (A × B)F1 (B + accessory) cells, whereas a mixture of these two Ts cell populations was able to significantly suppress the responses of F1 (B + accessory) cells. In contrast, responses of (A × B)F1 → parentA Th cells (restricted to recognizing parentA but not parentB MHC determinants on F1 cells) and (A × B)F1 (B + accessory) cells was suppressed by parentA-restricted Ts cells but not by parentB-restricted Ts cells. Collectively these findings suggest that the Ts cell populations characterized here do not function by directly inhibiting the activity of Th cells, B cells or accessory cells of a given MHC genotype, but rather that they appear to function through a unique mechanism involving highly specific inhibition of the interaction between MHC-restricted Th cells and the (B + accessory) cells required for these responses.

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