# T-CELL REGULATION OF ANTIBODY RESPONSES: DEMONSTRATION OF ALLOTYPE-SPECIFIC HELPER T CELLS AND THEIR SPECIFIC REMOVAL BY SUPPRESSOR T CELLS*

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The existence of T lymphocytes which suppress IgG antibody responses has been well established in a variety of systems (1–6). At least three questions about these cells, however, remain to be answered: (a) Are suppressors and cooperators (helpers) different types of T cells, or is suppression only a different manifestation of helper T-cell activity? (b) Do suppressor T cells directly attack the B cells responsible for antibody production, or do they suppress indirectly, for example, by interfering with the interaction between B cells and helper T cells? (c) How do suppressor T cells recognize their target?

The allotype suppression system provides a useful model for attacking these problems. Highly active suppressor T-cell populations are induced in SJL × BALB/c hybrids by perinatal exposure to antibody to allotype (Ig-1b) determinants present on IgG antibody molecules. These suppressor cells are capable of completely and specifically preventing production of antibody that carries the allotypic determinants both *in situ* and in adoptive transfer assays.

Since the suppressor T cells also suppress allotype production by co-transferred cells from syngeneic nonsuppressed donors in the adoptive transfer assay, suppressor and target populations may be taken from different donors. Thus with a hapten-carrier adoptive secondary assay, independently derived populations of carrier-primed helper cells (Th),[1] hapten-primed B cells (B), and suppressor cells (Ts) may be isolated, manipulated, and combined in various experimental protocols to effectively study the interactions between these three basic components of the humoral antibody response.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; FACS, fluorescence-activated cell sorter; GAT, linear copolymer of glutamic acid, alanine, and tyrosine; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; Th, helper T cell(s); Ts, suppressor T cell(s); TsF, soluble suppressive factors (from suppressed spleen culture supernate(s)).

Previous studies with this system suggested that the mechanism of allotype suppression might involve the removal or inactivation of Th by Ts rather than a direct attack on B cells committed to production of the suppressed allotype (7). Titration of Th, Ts, and B cells in these experiments showed that Th activity is lost in proportion to the Ts dose, regardless of Th- or B-cell dose (7). This quantitative relationship indicates stoichiometric removal of Th by Ts.[2] It suggests that Th and Ts are functionally different and that Th are the target of Ts.

The studies presented here directly confirm this hypothesis. We show first that Ts and Th are different types of cells distinguished by their Ly surface antigens. Cantor and Boyse (8, 9) have recently shown that Th carry Ly1 but not Ly2 surface antigens, which places Th in a distinct T-cell subclass (Ly1) comprising roughly 30% of peripheral T cells. The Th studied here, consistent with this observation, are killed by treatment with Ly1 antisera (plus complement) but not with Ly2 antisera.

In contrast, Ts used in these studies belong to the Ly2-positive subclass. They are killed by exposure to anti-Ly2 plus complement (C) but not by exposure to anti-Ly1 plus C. Thus Ts fall within the same (Ly2) subclass Cantor and Boyse have shown to contain cytotoxic cells and in a different subclass from (Ly1) Th (8, 9).

The ability to selectively kill Ts with anti-Ly2 while leaving the Th population unharmed allows a direct experimental approach to determining whether suppression of Ig-1b allotype production is due to removal of Th activity. If Ts remove Th, then a completely suppressed mouse should have no detectable Th activity for the suppressed allotype, since the Ts present in the animal would be expected to remove Th on appearance. Therefore killing the Ts (by anti-Ly2 treatment) in spleen cells from carrier-primed suppressed animals should not unmask any memory Th activity capable of helping with Ig-1b hapten-primed B cells. This prediction is confirmed by the data presented here.

This evidence, coupled with the well-documented specificity of allotype Ts for Ig-1b responses (6), suggested an unexpected division among Th which help IgG responses: Since Ts suppress Ig-1b production but do not impair production of other IgG antibodies in the same animal, the target Th must be dedicated to help only those B cells destined to produce Ig-1b antibody; Th which help other IgG B cells must be unable to help the Ig-1b response. Such Th specificity is unprecedented. Kishimoto and Ishizaka have presented evidence (10) indicating that IgE Th show restricted class specificity, but no evidence exists for allotype-specific help. In the studies presented here, however, we show directly that in the strain combination we use, Th which help Ig-1b B cells do not help other B cells and vice versa.

## Materials and Methods

Most of the methods and materials used for studies presented here are described in detail in an accompanying publication (11). The following briefly summarizes these methods and adds others unique to studies in this publication.

*Mice.* A new mouse strain, SJA/Hz congenic with SJL/JHz but carrying the BALB/c (Ig$^a$) chromosome region was mated with BALB/c to obtain Ig$^a$ homozygous hybrids (SJA × BALB/c) congenic with the (Ig$^b$/Ig$^a$) heterozygous (SJL × BALB/c)F$_1$ hybrid to SJL, selecting for progeny carrying the Ig$^a$ chromosome region at each successive backcross. Mice used here for mating (SJA/9) were from the third and fourth generations of an inbred line started with ninth backcross generation progeny.

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[2] The suppressed response is accurately predicted by the equation: response/B $= k(\text{Th} - \alpha \cdot \text{Ts})$; where B, Th, and Ts are given as the number of spleen cells transferred from the appropriate donor and $k$ and $\alpha$ are empirically determined scaling constants (7). The equation is valid so long as residual Th activity, i.e., Th $-$ Ts, does not exceed saturating Th levels.

*Allotype Suppressed Donors.*   All suppressed donors were (SJL × BALB/c)F₁ mice exposed perinatally to maternal (BALB/c) anti-Ig-1b. Donors were generally over 6 mo of age and always tested for Ig-1b just before transfer. Only donors showing no serum Ig-1b detectable by immunodiffusion (<0.01 mg/ml) were used.

*Priming.*   Mice were primed with 100 μg of 2,4-dinitrophenyl (DNP) keyhole limpet hemocyanin (KLH) on alum (hapten priming) plus 2 × 10⁹ of heat-killed *Bordetella pertussis* (kindly supplied by American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y.) at least 6 wk before use as donors in adoptive transfer. KLH (carrier)-primed mice received 100 μg KLH on alum plus 2 × 10⁹ *B. pertussis* 7 days before use as donors in adoptive transfer.

*Adoptive Transfer and Plaquing.*   Spleen cells from various donors were suspended in minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) and mixed at appropriate doses just before intravenous injection into BALB/c recipients irradiated (600 R) 18 h previously. Recipients were challenged at time of transfer with 10 μg aqueous DNP-KLH and sacrificed 7 days later for determination of DNP plaque-forming cells (PFC) in spleens. DNP-PFC were measured in Cunningham chambers (12). Indirect DNP-PFC were measured by determining the increase in DNP-PFC in chambers containing the appropriate facilitating antiserum over the response in chambers with no facilitating antisera (direct DNP-PFC). Results are expressed as DNP-PFC/10⁶ recipient spleen cells. Spleen size in adoptive recipients did not vary substantially.

*PFC Developing Antisera.*   Sera used to develop Ig-1a, Ig-1b, and total IgG were prepared as previously described (11). Alloantisera (BALB/c anti C57BL/6 allotype) used to develop Ig-4b DNP-PFC also contained anti-Ig-1b activity; therefore, Ig-4b response was determined by taking the difference between the number of DNP-PFC developed with this antiserum and the number of DNP-PFC developed with a specific anti-Ig-1b. The Ig-4b anti-DNP response determined in this fashion was completely blocked by addition of purified Ig-4b myeloma protein (MOPC-245).

Sera used to develop Ig-4a DNP-PFC were prepared by absorption of an SJL anti-BALB/c allotype serum onto S-8 (Ig-4a) myeloma protein bound to Sepharose, elution of the absorbed antibody, and passage through a Sepharose RPC-5 (Ig-1a) column to remove contaminating antibody. Specificity of all sera was tested in radioimmune assay.

*T-Enriched Spleen Cells by Nylon Wool Passage.*   Spleen cells were passed through nylon wool columns as previously described (13). Between 20 and 30% of spleen cells were recovered after passage. The recovered cells had greater than 95% T cells but less than 5% B cells (Ig bearing).

*Soluble Suppressive Factor (TsF) from Culture Supernates.*   Spleen cells from suppressed donors suspended in "Click" medium (14) were incubated for 48 h at 37°C in 5% CO₂ in air (2 × 10⁷ spleen cells in 1 ml of medium/6 cm Falcon tissue culture dish). After incubation cells were spun for 10 min at 290 g to pellet cells. Supernates were then removed and passed through a Swinnex filter (Millipore Corp., Bedford, Mass.) (0.22 μm) to remove residual cells. Spleen cells from nonsuppressed animals were similarly treated to provide control supernates. As an additional control, medium without cells was carried through the entire procedure.

*Incubation with Suppressive Supernatant Factor.*   1 ml of supernate prepared as above was added to 1 ml of fresh medium containing 10⁷ KLH-primed spleen cells from nonsuppressed donors. These suspensions were then incubated as above for 24 h after which the treated cells were harvested by centrifugation, washed three times with MEM (no fetal calf serum), resuspended, and tested for cooperator T-cell activity in the adoptive transfer assay. Harvested cells were between 50 and 70% viable in individual experiments.

*Antisera.*   Antisera to Ly antigens were prepared (and absorbed) as previously described (15). Anti-Ly1.2 was made by immunizing C3H/An with CE/J thymocytes, anti-Ly2.2 by immunizing (C3H/An × Bb-Ly2.1)F₁ with Bb leukemic cells ERLO, and anti-Thy1.2 by immunizing (A.Thy-1ᵃ × AKR-H-2ᵇ)F₁ with A-strain leukemia ASLI.

*Cytotoxic Treatment.*   Spleen cell suspensions were incubated with Ly1.2 and Ly2.2 or Thy1.2 cytotoxic antisera as previously described (reference 8 and footnote 3). Anti-Ly1.2, anti-Ly2.2, and anti-Thy1.2 were all used at a 1:20 final dilution. Rabbit C was used at a 1:10 or 1:15 final dilution. The Ly phenotype of (SJL × BALB/c)F₁ hybrid mice used in these studies is Ly-1.2,2.2,3.2; their Thy-1 phenotype is Thy-1.2.

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³ Huber, B., O. Devinski, R. K. Gershon, and H. Cantor. 1976. Delayed type hypersensitivity is mediated by a subclass of T cells. Manuscript in preparation.

TABLE I
Ly Phenotype of Ts

| (SJL × BALB/c)F₁ spleen cells transferred (× 10⁶) | | | | Indirect DNP-PFC* | | |
|---|---|---|---|---|---|---|
| Exp. no. | DNP-KLH-Primed (Th + B) | Suppressed Ts | | Ig-1b | Ig-1a | Total IgG |
| | | No. treated | Cytotoxic treatment‡ | | | |
| 1 | 10 | — | — | 220 | 380 | 2,760 |
| | 10 | 5 | NMS | 0 | 210 | 2,020 |
| | 10 | 5 | Anti-Ly1.2 | 0 | 320 | 2,810 |
| | 10 | 5 | Anti-Ly2.2 | 140 | 180 | 2,100 |
| 2 | 10 | — | — | 1,140 | 1,240 | 12,220 |
| | 10 | 5 | NMS | 40 | 1,130 | 10,220 |
| | 10 | 5 | Anti-Ly2.2 | 1,080 | 1,220 | 10,620 |
| | 10 | 5 | Anti-Ly2.2 (absorbed by Ly2.2 cells) | 0 | 990 | 9,120 |
| | 10 | 5 | Anti-Thy1.2 | 1,170 | 1,210 | 10,720 |

* Indirect DNP-PFC/10⁶ recipient spleen cells. Direct DNP-PFC (exp. 1, <50; exp. 2, <200) subtracted.

‡ Cells treated with indicated serum plus C. For details see Materials and Methods section. Number of cells transferred = remainder after treatment of indicated cell number.

## Results

*Allotype Ts Belong to the Ly2 T-Lymphocyte Subclass.* The data in Table I show that Ts belong to the Ly2⁺Ly1⁻ T-cell subclass [as defined by Cantor and Boyse (8, 16)]. All Ts activity, measured as suppression of the Ig-1b allotype-adoptive secondary DNP response mounted by co-transferred syngeneic, non-suppressed DNP-KLH-primed spleen cells, is completely removed from spleen cell suspensions of suppressed mice by cytotoxic pretreatment with antibody to Ly2.2. Cytotoxic treatment of the same cells with normal mouse serum, or with antibody to Ly1.2 determinants expressed on Th cells in the same strain (see below), does not impair suppressive activity, nor does treatment with anti-Ly2.2 previously absorbed with Ly2.2-bearing cells congenic to the antiserum donor. The selective killing of Ts by anti-Ly2.2 but not Ly1.2 shows that Ts belong to the Ly2-bearing subclass of T lymphocytes.

Data in Table II show that Th in SJL × BALB/c, like Th in other strains (8, 16) belong to the Ly1 subclass. Th activity, measured as the ability to help syngeneic hapten-primed B cells (T-depleted spleen cells) to mount an adoptive secondary DNP response, is completely removed by cytotoxic pretreatment with anti-Ly1.2 but not with anti-Ly2.2. Data in the second experiment shown in Table II demonstrate that Ly2.2-treated Th tested at a dose where Th limit the response still show no effect of anti-Ly2.2 treatment. Thus these cells carry Ly1.2 but not Ly2.2 and therefore belong to the Ly1 subclass.

*Genetically Determined Specificity of T-Cell Cooperation for Production of Allotype-Marked Antibody.* As indicated earlier (see introduction), previous

TABLE II
*Ly Phenotype of Th*

| Exp. no. | (SJL × BALB/c)F₁ spleen cells transferred (× 10⁶) | | | Indirect DNP-PFC* | | |
|---|---|---|---|---|---|---|
| | DNP-KLH-primed B cells‡ | KLH primed | | Ig-1b | Ig-1a | Total IgG |
| | | No. treated | Cytotoxic treatment§ | | | |
| 1 | 5 | — | — | 10 | 0 | 120 |
| | 5 | 10 | NMS | 120 | 160 | 1,420 |
| | 5 | 10 | Anti-Ly1.2 | 10 | 20 | 210 |
| | 5 | 10 | Anti-Ly2.2 | 100 | 120 | 1,250 |
| 2 | 5 | — | — | <10 | <10 | 130 |
| | 5 | 4 | Anti-Ly2.2 | 210 | 240 | 2,440 |
| | 5 | 4 | NMS | 200 | 220 | 2,290 |
| | 5 | 8 | NMS | 320 | 360 | 3,960 |

* Indirect DNP-PFC/10⁶ recipient spleen cells, direct DNP-PFC (<40) subtracted.
‡ T Cells were depleted by treatment with anti-Thy-1 plus C.
§ Cells treated with indicated serum plus C. For details see Materials and Methods section. Number of cells transferred = remainder after treatment of indicated cell number.

work suggested that Ts suppress immunoglobulin allotype production by removing Th required for production of antibody carrying the suppressed allotype. The work presented here, which confirms this hypothesis, tests several of its key predictions. The most startling of these derives from the well-documented specificity of allotype suppression. It predicts a heretofore unrecognized specificity of Th for the immunoglobulin allotype commitment of the B cells with which they cooperate. While no precedent for such specificity exists, the studies presented in the following section provide clear evidence that Th populations capable of helping B cells committed to produce antibody carrying one parental allotype in an allotype heterozygote do not help B cells from the same donor which are committed to production of antibody carrying the allelic allotype.

For these studies, we use memory B cells from hapten (DNP)-primed $Ig^b/Ig^a$ (SJL × BALB/c) hybrids. These donors are heterozygous for the $C_H$-chain allotypes specified by alleles at closely linked loci in the Ig chromosome region (17). They receive the $Ig^b$ chromosome region from SJL and the $Ig^a$ from BALB/c. Since the alleles in this chromosome region are co-dominantly expressed, the heterozygote produces both parental allotypes at each locus.

Priming these $Ig^b/Ig^a$ heterozygotes with DNP-KLH generates a number of distinct populations of hapten-primed memory B cells, each committed with respect to class and allotype. The relevant subsets of these memory B cells relevant for these studies are: Ig-1b and Ig-1a (class $\gamma G_{2a}$), and Ig-4a and Ig-4b (class $\gamma G_1$). Priming with DNP-KLH also generates Th capable of helping with each of these DNP memory B-cell subsets. The data in Table III show that in the adoptive secondary response to DNP-KLH, spleen cells from DNP-KLH-primed SJL × BALB/c hybrids give rise to both $\gamma G_{2a}$ and $\gamma G_1$ DNP-PFC, the $\gamma G_{2a}$ response being roughly one-quarter the $\gamma G_1$ response. Within each of these

TABLE III

*Failure of $Ig^a/Ig^a$ Th to Help Ig-1b Memory B Cells*

| Ig congenic spleen cells transferred ($\times 10^6$)* | | | Indirect DNP-PFC‡ | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | DNP-KLH-primed | KLH-primed nylon-passed T‖ | | | | | |
| Activity tested | $Ig^b/Ig^a$ B cells§ | $Ig^b/Ig^a$ Th | $Ig^a/Ig^a$ Th | Ig-1b | Ig-1a | Ig-4b | Ig-4a | Total IgG |
| | 5 | | | <10 | <10 | 40 | 40 | 150 |
| Th | 5 | 2.5 | | 160 | 170 | 600 | 650 | 1,930 |
| | 5 | 5 | | 310 | 300 | 1,000 | 1,100 | 3,470 |
| | 5 | 10 | | 380 | 410 | 1,240 | 1,320 | 4,140 |
| | 5 | | 2.5 | 20 | 180 | 560 | 510 | 1,620 |
| | 5 | | 10 | <10 | 350 | 860 | 980 | 3,110 |
| | 5 | | 30 | 20 | 320 | 740 | 870 | 3,230 |
| Ts in $Ig^a/Ig^a$ donor | 5 | 2.5 | 10 | 120 | 260 | 920 | 1,270 | 3,030 |
| | 5 | 2.5 | 30 | 120 | 230 | 1,220 | 1,190 | 3,320 |

* $Ig^b/Ig^a$ donors were (SJL $\times$ BALB/c)F$_1$; $Ig^a/Ig^a$ donors were (SJA $\times$ BALB/c)F$_1$.
‡ Indirect DNP-PFC/$10^6$ recipient spleen cells. Direct DNP-PFC (<50) subtracted.
‖ Nylon wool column purified T cells were used as source of Th.
§ T cells were depleted by treatment with anti-Thy-1 plus C.

classes, the number of DNP-PFC for each of the two parental allotypes (e.g., Ig-1a and Ig-1b) is essentially equal.

To compare Th from different sources for ability to help B-memory cells, the T cells from the DNP-KLH-primed heterozygous B-cell donor spleen must be removed by treatment with anti-Thy-1 before transfer so that all anti-DNP production will be dependent upon the carrier-primed Th being tested. As the data in Table III show, anti-Thy-1 treatment of these spleen cells before transfer abolishes the response. The response, however, is completely restored by syngeneic (SJL $\times$ BALB/c)F$_1$ KLH-primed splenic T cells, confirming that heterozygous mice have Th capable of cooperating with all four types of memory B cells.

In sharp contrast, the response is not completely restored when the Th from the carrier-primed $Ig^b/Ig^a$ heterozygotes are replaced with similarly primed Th from congenic (SJA $\times$ BALB/c)F$_1$ $Ig^a/Ig^a$ homozygotes. Th from the homozygotes restore only the Ig-1a and IgG$_1$ response. They do not restore the Ig-1b response. Even at 12 times the optimal dose (30 $\times 10^6$) of nylon-passed T cells, (which is equivalent to approximately 60 $\times 10^6$ spleen cells), no Ig-1b DNP-PFC are produced (see Table III). Thus although Th from $Ig^a/Ig^a$ homozygotes do help hapten-primed B cells from $Ig^b/Ig^a$ heterozygotes, as is shown by restoration of the Ig-1a and IgG$_1$ response, the $Ig^a/Ig^a$ homozygous Th are unable to interact with Ig-1b memory B cells to produce an Ig-1b response.

This failure of Th from $Ig^a$ homozygous donors to help Ig-1b memory B cells is not due to the presence of Ig-1b Ts which suppress Ig-1b memory cell expression, since 10 or 30 million SJA $\times$ BALB/c T cells co-transferred with a limiting dose of heterozygous Th gives essentially the same Ig-1b response as that number of

TABLE IV

*Absence of Ig-1b Th Activity in Ig-1b Suppressed Mice: Ts Removed by Anti-Ly2.2*

| (SJL × BALB/c)F₁ spleen cells transferred (× 10⁶) | | | | | Indirect DNP-PFC* | | |
|---|---|---|---|---|---|---|---|
| Activity tested | DNP-KLH-primed B cells‡ | KLH-primed Th | KLH-primed suppressed (Th + Ts enriched)§ | | Ig-1b | Ig-1a | Total IgG |
| | | | No. treated | Treatment‖ | | | |
| | 6 | | | | <10 | <10 | 20 |
| | 6 | 6 | | | 290 | 310 | 3,050 |
| Th | 6 | 4 | | | 200 | 250 | 1,880 |
| | 6 | | 4 | NMS | <10 | 370 | 3,790 |
| | 6 | | 4 | Anti-Ly2.2 | <10 | 300 | 3,220 |
| Ts | 6 | 2 | | | 100 | 130 | 900 |
| | 6 | 2 | 2.5 | Anti-Ly2.2 | 120 | 300 | 3,000 |
| | 6 | 2 | 2.5 | NMS | <10 | 370 | 3,690 |

\* Indirect DNP-PFC/10⁶ recipient spleen cells. Direct DNP-PFC (<40) subtracted.

‡ T cells were depleted by treatment with anti-Thy-1 plus C.

§ B cells were depleted from spleen cell population by nylon wool passage before treatment. T-enriched population had more than 80% T cells and less than 5% B cells (13).

‖ Cells treated with indicated serum plus C. For details see Materials and Methods section. Number of cells transferred = remainder after treatment of indicated cell number.

heterozygous Th transferred alone (see Table IV). The dose of heterozygous Th used here is set considerably below the saturating Th dose, making this assay highly sensitive for detecting Ts in the homozygous donors. Therefore, the absence of the Ig-1b response when carrier-primed Igᵃ/Igᵃ homozygotes are used as donors must be due to a genetically determined absence of Th (or Th activity) capable of helping Ig-1b B cells.

Th capable of helping with IgG memory cells also show specificity with respect to the B cells which they help. Although the Igᵃ/Igᵃ homozygous populations help both Ig-4a and Ig-4b (IgG₁ allotypes) the absence of an Ig-1b response in the presence of these Th shows that they do not help Ig-1b memory cells. Thus, at least in the (SJL × BALB/c)F₁ and (SJA × BALB/c)F₁ congenic pair, Th show specificity for the immunoglobulin commitment of the B cells which they help. This demonstration, then, clears the way for consideration of a mechanism of suppression based on the selective removal of Th capable of helping Ig-1b B cells.

*Absence of Ig-1b Th Activity in Carrier-Primed Suppressed Mice.* Before testing for Ig-1b Th activity in spleen cell suspensions from suppressed mice, Ts must be removed. Otherwise, the Ig-1b Th may be masked by the Ts. Demonstrating the complete depletion of Ts, however, is complicated by the fact that the Ig-1b response in adoptive transfer recipients of Th and Ts is determined by the difference between the amounts of Th and Ts present, i.e., DNP-PFC/B = $k(\mathrm{Th} - \alpha \cdot \mathrm{Ts})$ (7). There is no problem if Ts exceeds Th, even by a small amount, since the suppressive activity is then detectable by transferring with a low dose of nonsuppressed Th (and hapten-primed B cells) which makes the assay highly

sensitive for Ts. If Th exceeds Ts, again there is no problem because the Th will be detectable by transferring with hapten-primed B cells despite Ts presence. But if the amounts of Ts and Th in the test cell suspension are sufficiently close so as to simply neutralize one another, neither Ts nor Th will be detectable and the result will be the apparent absence of Th. To overcome this problem, we used two quite different methodologies for selectively depleting the Ts from the KLH-primed suppressed donors: killing, in the presence of C, with antiserum to Ly2 surface determinants and size separation with the fluorescence-activated cell sorter (FACS) (18).

Data presented earlier with the anti-Ly2 antiserum showed that Ts are killed by treatment with the antiserum and C but that Th in KLH-primed nonsuppressed spleen are not (see Tables I and II). Therefore, spleen cells from KLH-primed Ig-1b-suppressed mice were treated with anti-Ly2 and C and the surviving cells assayed for Th with hapten-primed B cells in the DNP-adoptive secondary assay.

Results from these studies are presented in Table IV. The inability of treated cells to suppress Ig-1b DNP-PFC formation when co-transferred with a low dose of Th from a nonsuppressed primed donor shows the complete removal of Ts by anti-Ly2 treatment. Less than 5% of the original Ts activity is detectable. However, no Ig-1b are unmasked. KLH-primed suppressed spleen cells still are unable to help Ig-1b memory cells when Ts are gone, although, as the data show, Ig-1a and $\gamma G_1$ (total IgG) Th are unharmed by the treatment with anti-Ly2.

Treatment and transfer of 10 million KLH-primed suppressed spleen cells showed similar results, i.e., complete removal of Ts activity but no detectable Ig-1b help. Since, in the assay used here, Ts activity in 0.3 million spleen cells from unprimed suppressed animals is adequate to significantly suppress the Ig-1b response mounted by the co-transferred nonsuppressed primed cells (7), treatment with anti-Ly2 could leave no more than 3% of the original Ts activity in the treated cell population. This residual Ts activity would be far too small to suppress the response due to masked Ig-1b Th in the primed suppressed donor if these Th were present in the same numbers as in primed nonsuppressed donors.

Similar results are obtained when Ts are removed by size separation (measured by a light-scattering parameter) with the FACS. Preliminary experiments showed that Ts in unprimed suppressed mice are confined to the FACS-separated fraction containing the largest 20% of splenic T cells. Th in the splenic T-cell suspension, however, are found in both large and small cell fractions so that, as the data in Table V show, when splenic T cells from carrier-primed suppressed mice are separated by size with the FACS, a substantial portion of the Th for Ig-1a and $\gamma G_1$ are found in the small cell fraction. No Ig-1b Th are found in this fraction, however, despite the demonstration that the fraction has no apparent suppressor T-cell activity (see Table V).

Thus, since spleen cell suspensions from KLH-primed suppressed mice have no Ig-1b Th activity when tested after Ts have been removed by two independent methods, we feel reasonably safe in concluding that Ts specifically remove Ig-1b Th activity in intact primed suppressed animals. This conclusion is supported by evidence presented in the following section which shows that pretreatment of spleen cells from KLH-primed, nonsuppressed mice with supernates from cul-

TABLE V

*Absence of Ig-1b TH Activity in Ig-1b Suppressed Mice: Removal of Ts by Size Separation with FACS*

| Activity tested | DNP-KLH primed | | KLH-primed suppressed (Th + Ts enriched)‡ | | Indirect DNP-PFC* | | |
| | (Th + B) | B Cells§ | Fraction | No. | Ig-1b | Ig-1a | Total IgG |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | 4 | | | <10 | <10 | 20 |
| Th | | 4 | Unseparated | 1.5 | <10 | 750 | 7,020 |
| | | 4 | Small‖ | 1.5 | <10 | 310 | 3,240 |
| Ts | 6 | | | | 780 | 880 | 9,380 |
| | 6 | | Unseparated | 1.5 | 10 | 850 | 8,910 |
| | 6 | | Small‖ | 1.5 | 800 | 900 | 9,420 |

Header spanning columns: (SJL × BALB/c)F₁ spleen cells transferred (× 10⁶) covers the first four data columns; Indirect DNP-PFC* covers Ig-1b, Ig-1a, Total IgG.

* Indirect DNP-PFC/10⁶ recipient spleen cells. Direct DNP-PFC (<100) subtracted.
‡ See footnote §, Table IV.
§ T cells were depleted by treatment with anti-Thy-1 plus C.
‖ Small cell fraction (smallest 70%) was separated by low-angle light scatter with the FACS. For details of separation and transfer, see Materials and Methods section.

tures of unprimed suppressed spleen cells specifically removed Th activity capable of helping Ig-1b B cells.

*Ig-1b Helper T-Cell Activity Removal by In Vitro Treatment with Cell-Free Culture Supernate (Factor) from Suppressed Spleen Cells.* The Th capable of helping Ig-1b memory cells are selectively removed from KLH-primed SJL × BALB/c spleen cells by culturing these cells for 24 h with culture medium in which suppressed unprimed spleen cells were first cultured for 48 h. The ability of treated Th to help Ig-1a and γG₁ memory B cells in the adoptive transfer assay is unaffected by the treatment (see Table VI).

Only the TsF is able to deplete the Ig-1b Th. Supernates from nonsuppressed spleen cultures or from culture dishes with no cells added have no effect on Ig-1b help (also Table VI).

Induction of suppressor cells in the TsF-treated carrier-primed spleen, which could subsequently suppress Ig-1b production in the adoptive transfer assay, was ruled out by transferring the TsF-treated cells together with a low dose of untreated carrier-primed spleen (as in the preceding experiment). The data in Table VI show that the TsF-treated cells have no Ts activity. Ig-1b responses were the same with or without addition of the treated cells.

The absence of Ts in the treated Th cultures was further substantiated by exposing the TsF-treated Th to anti-Ly2.2 plus C before testing in adoptive transfer. If Ts had been induced by exposure to TsF these Ts should have been killed by the anti-Ly2.2 and the Th activity of the treated culture restored. As the data in Table VII show, however, Th activity in the TsF-treated cultures was still absent after Ly2.2 treatment. Similar results are obtained if the KLH-primed cells are treated with Ly2.2 before exposure to TsF. These data suggest

TABLE VI

*Removal of Ig-1b Th Activity by Treatment with TsF*

| | (SJL × BALB/c)F₁ spleen cells (× 10⁶) | | | | Indirect DNP-PFC* | | |
|---|---|---|---|---|---|---|---|
| | | KLH-primed | | | | | |
| Activity tested | DNP-KLH-primed T-depleted B‡ | Un-treated Th | Treated§ | | Ig-1b | Ig-1a | Total IgG |
| | | | Num-ber | Factor Source | | | |
| | 5 | | | | <10 | <10 | <10 |
| Th | 5 | | 10 | Suppressed | 20 | 280 | 3,420 |
| | 5 | | 10 | Nonsuppressed | 250 | 260 | 2,720 |
| | 5 | | 10 | Medium alone | 220 | 250 | 2,440 |
| Ts | 5 | 2.5 | | | 90 | 120 | 1,120 |
| | 5 | 2.5 | 10 | Suppressed | 250 | 260 | 2,830 |

* Indirect DNP-PFC/10⁶ recipient spleen cells. Direct DNP-PFC (<20) subtracted.

‡ T cells were depleted by treatment with anti-Thy-1 plus C.

§ Culture supernates were obtained by culturing spleen cells of indicated type for 48 h. KLH-primed cells were incubated for 24 h with culture supernates, then washed and tested for Th and Ts activity in adoptive transfer. Number of cells transferred = remainder after treatment of indicated cell number (see Materials and Methods).

TABLE VII

*Failure of (TsF) to Generate Ts In Vitro*

| (SJL × BALB/c)F₁ spleen cells (× 10⁶) | | | Indirect DNP-PFC* | | |
|---|---|---|---|---|---|
| DNP-KLH-primed T-depleted B‡ | KLH-primed TsF incu-bated§ | | Ig-1b | Ig-1a | Total IgG |
| | No. treated | Cytotoxic treatment‖ | | | |
| 5 | — | — | <10 | <10 | <10 |
| 5 | 10 | NMS | 10 | 230 | 1,220 |
| 5 | 10 | Anti-Ly2.2 | 0 | 260 | 1,230 |

* Indirect DNP-PFC/10⁶ recipient spleen cells. Direct DNP-PFC (<20) subtracted.

‡ T cells were depleted by treatment with anti-Thy-1 plus C.

§ KLH-primed spleen cells were incubated with TsF.

‖ For details of treatment and adoptive transfer, see Materials and Methods section. Cells treated with indicated serum plus C. Number of cells transferred = remainder after treatment of indicated cell number.

that Th activity for Ig-1b is specifically removed by TsF treatment rather than masked by the induction of Ts during the culture.

## Discussion

In recent years, a number of cases have been studied where nonresponsiveness occurs because T-cell populations are present which actively suppress

antibody production (1–6). We have shown here that in one such system (allotype suppression), the Ts remove Th activity and thus regulate antibody formation by limiting the amount of available Th activity.

We first demonstrated that Ts and Th are different types of T cells which belong to different T-cell subclasses. This was accomplished by showing that Ts carry Ly2 and not Ly1 surface antigens, thus placing Ts within the same $Ly2^+Ly1^-$ T-cell subclass as cytotoxic precursor and effector cells (8, 9). Th, in contrast, express Ly1 and not Ly2 surface antigens and thus belong to the $Ly1^+Ly2^-$ T-cell subclass which helps both humoral (8, 16) and cytotoxic responses (8, 9) and can initiate delayed hypersensitivity reactions.[3]

These findings have considerable bearing on possible mechanisms of suppression. Since cells of the Ly2,3 subclass do not show helper activity, the identification of Ts as belonging to this subclass makes highly unlikely the suggestion that Ts populations suppress by providing an excess of helper activity.[3] Furthermore, since Huber and Cantor have shown that T cells in the Ly1 subclass do not convert to Ly2-positive cells,[4] the data presented here make it unlikely that Ts are modified Th. Instead, these data suggest that Ts and Th are distinct differentiated populations, each with its own role in regulation of the antibody production.

The difference in these roles is shown directly by our studies on the mechanism of suppression. Th are required to help B cells to increase in number and differentiate to antibody-forming cells (AFC). Therefore, Th exert direct control over IgG antibody production. Ts, on the other hand, regulate antibody production indirectly. They remove Th activity and thus reduce the amount of Th activity available to help B cells.

The conclusion that Ts remove Th is based on evidence presented here which shows (a) that carrier-primed allotype suppressed mice have no demonstrable Th activity capable of helping Ig-1b B cells; and (b) that cultured spleen cells from suppressed mice produce soluble factors which interact with carrier-primed nonsuppressed spleen cells to specifically deplete Ig-1b Th activity. Taken together, these studies strongly suggest that Ts exert a direct effect on Th rather than on Ig-1b B cells. We support this conclusion with direct evidence showing that, at least in the strain combination used here, Ig-1b B-memory cells require Ig-1b-specific Th which cannot be replaced by Th which help Ig-1a or $IgG_1$ B cells. This demonstration is required to explain how allotype Ts can specifically suppress Ig-1b antibody production without interfering with Ig-1a or other IgG production in allotype suppressed mice.

In the accompanying publication (11), we have presented evidence showing that priming and persistence of Ig-1b memory B cells is unimpaired in hapten-primed allotype-suppressed mice. T-depleted spleen cell suspensions from these mice (supplemented with carrier-primed spleen cells from nonsuppressed mice) show the same response in an adoptive secondary assay as supplemented T-depleted spleen cells from hapten-primed nonsuppressed mice. Thus the inability of intact, allotype-suppressed mice to produce Ig-1b antibody appears to be due solely to the removal of Ig-1b Th activity by Ts.

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[4] Huber, B., and H. Cantor. 1976. The developmental relationship between helper ($T_H$) and killer ($T_{CS}$) T cell subclasses. Manuscript in preparation.

The restrictions in inducing allotype suppression in other mouse strains (6) dictate caution in extending our findings to other suppressor systems; nevertheless, there are some suggestions that our findings that Ts suppress by removing Th activity may reflect a general immunoregulatory mechanism. Tada has proposed that KLH Ts interfere with Th function because KLH-specific Ts-soluble factors suppress anti-hapten responses when the hapten is coupled to KLH as a carrier (3). Okumura and Tada's studies on suppression of IgE responses (19) may be similarly interpreted, especially since Kishimoto and Ishizaka have shown that IgE Th appear to be specific for IgE B cells (10). Kapp et al. (20) have shown that removal of Ts which suppress the response to GAT, (a linear copolymer of glutamic acid, alanine, and tyrosine) in GAT-suppressed nonresponder mice does not unmask GAT Th activity. These authors suggest that Th are missing because GAT does not prime T cells in GAT-suppressed mice (20), but it is also possible that the GAT Ts have removed the GAT Th. Thus, Th removal by Ts could prove to be a general mechanism of suppression of T-dependent responses.

In addition to the functional similarities listed above, several surface antigenic similarities exist between allotype Ts and other Ts. Ts in several other systems have now been shown to belong to the Ly2,3 subclass in contrast to Ly1 helper cells (16, 21). We have also shown recently that allotype Ts, like Ts generated in the A5A idiotype suppression system (22) and like soluble suppressive factors in Tada's KLH Ts system (23), carry Ia determinants (references 24 and 25, and footnotes 5 and 6). Data from Tada's and our studies indicate that these determinants map to a previously uncharted segment of the $I$ region between $I$-$B$ and $I$-$C$. Our studies indicate that this new region controls Ia antigens on T rather than B cells.[5,6]

Parallels between allotype and idiotype suppression, while not fully established, offer intriguing ground for speculation on how Ts recognize Th and how Th recognize B cells. Eichmann (26) has shown that exposure to antibody determinants (idiotypes) on immunoglobulin molecules results in the generation of a Ts population which specifically suppresses production of immunoglobulin molecules carrying that idiotype. This closely parallels the allotype-suppression system, where exposure to antibody to allotypic determinants on immunoglobulin generates Ts specific for allotype production.

There are, however, significant differences between allotype and idiotype suppression. Idiotypic determinants are in the variable region located in the Fab portion of the immunoglobulin molecule, while the Ig-1b allotypic determinants used in our studies are found on the Fc portion of the immunoglobulin H chain. Furthermore, idiotype Ts suppress production of idiotype-bearing antibody molecules in all immunoglobulin classes, while allotype Ts suppress production of antibody molecules carrying the Ig-1b allotype regardless of the specificity of the

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

[6] Murphy, D. B., L. A. Herzenberg, K. Okumura, L. A. Herzenberg, and H. O. McDevitt. 1976. A new $I$ subregion ($I$-$J$) marked by a locus ($Ia$-$4$) controlling surface determinants on suppressor T lymphocytes. J. Exp. Med. 144:in press.

antibody-combining site. These differences indicate that the two types of Ts affect the expression of different subsets of B cells; but whether the mechanism of suppression, i.e. helper depletion, is the same in both cases remains to be determined.

The demonstration that allotype Ts suppress B-memory expression by removing helper T cells establishes these three types of cells in an expanded network similar to that postulated by Jerne (27, 28). Idiotype and carrier-specific suppression also fit into similar networks. It is possible that these networks contain other cells as well, e.g. helper cells for the development of functional Ts and Th, precursors of Th, Ts, B, etc. (The allotype network could also contain two types of Th, both required for B-cell expression — one for carrier recognition and one for allotype recognition; this is entirely speculative but would avoid having to endow a single Th with the ability to recognize both types of determinants.) Such networks must then interlock with one another since allotype suppression stays within Ig class lines but cuts across antigen-specific responses, whereas idiotype and carrier-specific suppression do the opposite.

It is still too early to obtain a clear insight into the molecular basis of communication between cells in a given network. Tada has shown that carrier-specific suppressive factors (KLH-TsF) have both $I$-region and carrier recognition determinants (23). We have shown here that spleens from allotype-suppressed mice produce a soluble factor (allo-TsF) which interacts with carrier-primed spleen to remove allotype-specific Th. Since allotype Ts have surface $I$-region determinants which map quite close to the $I$-region determinants on KLH-TsF, it is quite possible that allo-TsF also carries $I$-region determinants. If so, then the communication between Ts and Th may generally utilize $I$-region determinants as part of a recognition mechanism. This would be consistent with the view that $I$-region determinants are involved in Th-B collaboration (29), although these determinants need not be the same as those involved in Ts-Th communication. Such considerations, however, do not address the heart of the recognition question posed by the mechanism of suppression presented here, i.e., how allotype Ts recognize Ig-1b Th and how Ig-1b recognize Ig-1b memory B cells. No data are currently available which bear on this point.

Thus far in this discussion we have considered the demonstration of allotype-specific help mainly within the context of the mechanism of allotype suppression. The implications of this unprecedented finding with respect to regulation of antibody production, however, deserve consideration in their own right. The data presented here show directly that Th capable of cooperating with Ig-1a B cells do not cooperate with Ig-1b B cells. The converse is also true, since removal of Ig-1b Th activity does not affect the Ig-1a response at limiting Th doses.

## Summary

Allotype suppressor T cells (Ts) generated in SJL × BALB/c mice specifically suppress production of antibodies marked with the Ig-1b allotype. The studies presented here show that allotype Ts suppress by specifically removing helper T cell (Th) activity required to facilitate differentiation and expansion of B cells to Ig-1b antibody-forming cells.

We show first that Ts and Th belong to different T-cell subclasses as defined by Ly surface antigens. Ts are Ly2$^+$Ly1$^-$ and thus belong to the same subclass as

cytotoxic precursor and effector cells; Th are Ly1$^+$Ly2$^-$ cells and thus belong to the subclass containing cells which can exert helper functions and initiate delayed hypersensitivity reactions. Placing these cells in these two subclasses shows that Th are different from Ts and suggests that they play different roles in regulating antibody responses. The difference in these roles is defined by the evidence presented here showing that Ts attack Th and regulate the antibody response by specifically regulating the availability of Th activity. We show that in allotype suppressed mice, Ts which suppress Ig-1b antibody production have completely removed the Th activity capable of helping Ig-1b B cells without impairing Th activity which helps other IgG B cells.

These findings imply the existence of allotype-specific Th for Ig-1b cells (Ig-1b Th). We directly establish that Ig-1b cells require such help by showing that carrier-primed spleen cells from Ig$^a$/Ig$^a$ congenic hybrids help Ig-1a B cells from hapten-primed Ig$^b$/Ig$^a$ donors but do not help Ig-1b B cells from the same donor in the same adoptive recipient.

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