ABSENCE OF THE Lyt-2⁻,L3T4⁺ LINEAGE OF T CELLS IN MICE TREATED NEONATALLY WITH ANTI-I-A CORRELATES WITH ABSENCE OF INTRATHYMIC I-A-BEARING ANTIGEN-PRESENTING CELL FUNCTION

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Experiments with radiation-induced bone marrow chimeras and thymus-reconstituted nude mice have shown that the repertoire of T cells is influenced greatly by the major histocompatibility complex (MHC)[1] genes expressed in their developmental milieu. For class I MHC–restricted T cells, there appear to be both thymic and extrathymic influences on the repertoire (1–8), while for class II MHC–restricted T cells, the I region gene phenotype of the thymus appears to determine entirely the self-restriction specificity (9–13). The precise mechanism by which the thymus shapes the T cell receptor repertoire is unclear, but it appears that bone marrow–derived, medullary, Ia-bearing, thymic, antigen-presenting cells (APC) play a critical role in determining class II MHC–restriction specificity (14–16).[2]

In these experiments, the process that generates the T cell repertoire is not readily accessible to experimental manipulation. The recent development of a model system in which mice are treated from birth with anti-I-A has allowed us to look at the process of T cell development more closely. In addition to its effects on B cell development (17), chronic anti-I-A treatment early in life has

[1] Abbreviations used in this paper: APC, antigen-presenting cell; BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; CTLL, IL-2-dependent line of cytotoxic T lymphocyte; E/R, Eagle's/RPMI special medium; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; NMS, normal mouse serum; PI, propidium iodide; TR, Texas Red.

[2] Bone marrow–derived thymic antigen presenting cells determine self-recognition of Ia-restricted T lymphocytes. D. L. Longo, A. M. Kruisbeek, M. L. Davis, and L. A. Matis. Manuscript submitted for publication.

profound effects on T cell development (18),[3] despite the fact that murine T cells do not express I-A. Thymic and splenic Ia-antigen expression is strongly reduced in mice treated neonatally with anti-I-A (17, 18). In vitro cytotoxic T lymphocyte (CTL) responses to alloantigens and trinitrophenyl–modified-self antigens by spleen cells from such mice could only be generated in the presence of an exogenous source of interleukin 2 (IL-2) (18), implying that T cells capable of generating helper factors in vitro are defective in anti-I-A–treated mice. Finally, recent studies[3] demonstrated abrogation of allo–class II–specific and self–class II–restricted T cell proliferative responses in both thymus and spleen of anti-I-A–treated mice. These data (18)[3] suggested that class II MHC–restricted T cells were either missing or functionally defective in mice treated from birth with anti-I-A.

In an effort to distinguish whether class II–restricted T cells were absent or defective in this model, we examined the effects of neonatal anti-I-A treatment on the L3T4 and Lyt-2 surface phenotype of thymic and splenic T cells, and on their class II–specific T cell functions. In addition, we tested the hypothesis that the T cell defects reported here and previously (18)[3] were somehow related to the reduced thymic Ia antigen expression (18) by assessing the relationship of the class II–specific T cell defects to the function of thymic APC. Identifying the L3T4 and Lyt-2 phenotype of T cells should be a reliable measure for presence or absence of a particular T cell subset because of the striking correlation between cell surface phenotype and MHC-subclass specificity. The expression of Lyt-2,3 molecules on mature mouse T cells usually correlates with class I MHC specificity (19–23), while expression of the L3T4 antigen correlates with class II MHC specificity (24–29). How this impressively strong association between T cell surface marker expression and MHC-subclass specificity is acquired in the developing T cell repertoire is unknown. In the thymus, all precursors of alloreactive IL-2-producing T cells are contained within the subset of phenotypically "mature" Lyt-2⁻,L3T4⁺ T cells (30), while all alloreactive CTL precursors are recovered in the Lyt-2⁺ subset of "mature" T cells (31).

We report here that the functional defects in class II–restricted T cells in anti-I-A–treated mice are correlated with an absence of the Lyt-2⁻,L3T4⁺ T cell subset, and with absence of functional thymic APC. The Lyt-2⁺,L3T4⁻ class I–restricted T cell subset develops normally in these mice. When anti-I-A treatment is stopped, thymic and splenic APC function return to normal, and the Lyt-2⁻,L3T4⁺, class II–restricted T cell subset reappears, first in the thymus, with return of these cells to the periphery lagging behind. Collectively, these findings suggest that development of Lyt-2⁻,L3T4⁺, class II–restricted T cells is dependent on I-A-bearing thymic APC, and that the Lyt-2⁺,L3T4⁻, class I–specific T cell subset develops by a distinct mechanism, unaffected by anti-I-A.

Materials and Methods

Mice and Anti-I-A Treatment.   Neonatal C57BL/6 (H-2ᵇ) mice were treated within 24 h of birth with protein A–purified anti-I-Aᵇ (hybridoma Y-3P [32], a kind gift from Dr.

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[3] In vivo treatment of neonatal mice with anti-I-A antibodies interferes with the development of the class I–, class II– and M ls-reactive proliferating T cell subset. 1985. A. M. Kruisbeek, S. Bridges, J. Carmen, D. L. Longo, and J. J. Mond. *J. Immunol.* In press.

Charles A. Janeway, Yale University, New Haven, CT) and treated every 48 h thereafter. Intraperitoneal injections of 600, 800, and 1,000 μg of antibody were given during the first, second, and third week of life, respectively. Mouse strains to be used as donors for stimulator cells were DBA/2, B6.C.H-2^{bm1}, D1.LP, B10.D2 (The Jackson Laboratory, Bar Harbor, ME) and B6.C.H-2^{bm12} (bred in our facilities from breeding pairs provided by Drs. Richard Hodes, NIH, and Ted Hansen, Washington University, St. Louis, MO). H-2 and M1s phenotypes of these strains are: DBA/2: H-2^d, M1s^a; B10.D2: H-2^d, M1s^b; B6.C.H-2^{bm1}: H-2^b (K^{bm1} mutant), M1s^b; D1.LP: H-2^b, M1s^a; B6.C. H-2^{bm12}: H-2^b (I^{bm12} mutant), M1s^b. Mice treated with anti-I-A were killed at 3 wk of age, either after continuous antibody treatment, or after 1 wk of treatment and 2 wk with no treatment.

Generation of T Cell Proliferative Responses in Mixed Lymphocyte Culture (MLC). Responder thymocytes were cultured at 6 × 10^5 cells/well, and spleen cells at 4 × 10^5 cells/well, with varying concentrations (2, 4, and 8 × 10^5) of 2,000 rad irradiated allogeneic stimulator spleen cells in a final volume of 0.2 ml of complete tissue culture medium consisting of half RPMI 1640, half Eagle's Hanks' amino acids medium (33) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml), 2-mercaptoethanol (5 × 10^{-5} M), and sodium pyruvate (0.11 mg/ml). This medium is referred to as E/R. Triplicate cultures were set up in round-bottom (thymocytes) or flat-bottom (spleen cells) 96-well microtiter plates. On day 4 for thymocytes or day 3 for spleen cells, 1 μCi of [^{3}H]thymidine (sp act 6.7 Ci/mmol; New England Nuclear, Boston, MA) was added per well, and cultures were harvested 16–18 h later on an automated sample harvester. [^{3}H]Thymidine incorporation was measured in a liquid scintillation counter, and data are expressed as the arithmetic mean cpm. SEM were <20%.

Fluorescence Staining and Two-color Fluorescence-activated Cell Sorter (FACS) Analysis. Spleen cell and thymocyte suspensions from normal and anti-I-A–treated mice were prepared in Hank's balanced salt solution (without phenol red) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (FACS buffer). Cells (10^6/30 μl buffer) were incubated on ice for 30 min with 10 μl of the appropriate reagent, and washed twice after each incubation. For two-color FACS analysis of L3T4 and Lyt-2, the following sequence was used for thymocytes: (*a*) anti-Lyt-2.2 culture supernatants (mouse IgG2a, clone 19/178 [34]); (*b*) Texas Red (TR)-labeled, affinity-purified goat anti–mouse Ig (crossreactive with rat Ig) (35); (*c*) normal mouse serum (NMS); (*d*) anti-L3T4 culture supernatant (rat IgG2b, clone GK-1.5 [24, 27]); (*e*) fluorescein isothiocyanate (FITC)-conjugated goat anti–rat IgG (not crossreactive with mouse IgG) (Cappel Laboratories, Cochranville, PA). The sequence used for spleen cells was (*a*) anti-L3T4 culture supernatant; (*b*) FITC-conjugated mouse anti–rat κ (mouse IgG2a, clone Mar18.5 [36]); (*c*) rat Ig fraction (purified from rat serum); (*d*) biotin-labeled anti-Lyt-2 (rat IgG2a, clone 53-6.7 [37] purified by ion-exchange and gel filtration); or (*e*) TR-avidin. NMS or rat Ig, which had the capacity to react with both primary antibodies, was used to block unoccupied binding sites on bound second antibody. Control cells, either unstained or stained with second antibody alone, were used to obtain background fluorescence levels. In later experiments, not shown here, we used, with equal success, the following sequence for both thymocytes and spleen cells: (*a*) biotin-labeled anti-L3T4; (*b*) TR-avidin; (*c*) FITC-conjugated anti-Lyt-2.

Flow microfluorometry analysis was performed on a FACS II (Becton-Dickinson and Co., Mountain View, CA), and analyzed by a PDP 11/34 computer (Digital Equipment, Marlboro, MA), using programs previously described (38). All data were collected using log amplifiers and displayed on a log$_{10}$ scale of increasing green and red fluorescence intensity, without defining units. Subpopulations in stained samples that did not differ from the unstained control were scored as negative. Dead cells were rejected from analysis by forward light scatter (39) and propidium iodide staining (PI) by electronic gating. 10 μl of PI at 50 mg/ml in 0.1% sodium citrate (40) were added to the cell sample just prior to FACS-analysis.

Generation of IL-2 in Limiting-dilution Microculture. A modification of the method described by Miller and Stutman (41) was used. Responder C57BL/6 spleen cells or

thymocytes were, in some experiments, depleted of Lyt-2-bearing cells by anti-Lyt-2.2 monoclonal antibodies (mAb) and complement treatment to eliminate cells that may use IL-2 (42). Stimulator cells were nonirradiated resident peritoneal cells from C57BL/10 × B10.D2 F$_1$ nude mice or C57BL/6 × bm12 F$_1$ mice treated wtih anti-Thy-1.2 mAb plus complement to eliminate T cells; these stimulator cells were shown to be devoid of T cells by their inability to generate a concanavalin A–induced proliferative response (data not shown). Varying numbers of untreated or Lyt-2-depleted responder cells were cultured in round-bottom wells of 96-well microtiter plates with 1–5 × 10^4 F$_1$ stimulator cells for 5 d. After centrifugation of the plates at 500 g, 100 μl of supernatant was removed from each well, and tested for its IL-2 activity by measuring its ability to support growth of CTLL cells (an IL-2-dependent CTL line). The supernatants to be tested were cultured with 2 × 10^3 CTLL cells in flat-bottom wells of 96-well microtiter plates. After 24 h, cultures were labeled with 1 μCi of [^{3}H]thymidine for 4–6 h, and harvested and processed as described above for MLC cultures. Supernatants of microcultures were defined as positive when the IL-2 activity exceeded by >3 SD the mean [^{3}H]thymidine incorporation of CTLL cells grown in control supernatants (i.e., obtained from 5-d cultures of stimulator cells only). Minimal estimates of the precursor frequencies were calculated by plotting the percent negative cultures against cell dose, according to the Poisson model, and by using the minimum χ^2 method of estimating the frequency, as described by Taswell (43).

Measurement of APC Function for I-region–specific T Cells with Antigen-reactive T Cell Clones.   Antigen-reactive T cell clones were derived from draining lymph nodes of mice immunized with keyhole limpet hemocyanin (KLH) (lot 230145, Calbiochem-Behring Corp., San Diego, CA) 8–10 d earlier (50 μg KLH per hind foot pad in an emulsion with complete Freund's adjuvant) as previously described (44). The cells were cloned by limiting dilution 2 d after the third stimulation with antigen and syngeneic 3,300 rad irradiated spleen cells. Alloreactive T cell clones were derived from secondary MLC; both antigen-specific and alloreactive T cell clones were maintained by alternating 4-d periods of stimulation and 7-d periods of rest with syngeneic irradiated spleen cells. I-region specificity was determined by mapping using recombinant mouse strains, and by inhibition of proliferation with mAb of the appropriate specificity. All clones were independent of exogenous IL-2 for their proliferation.

T cell clones recovered at the end of the rest period were used to assay APC function of thymocytes or spleen cells from control or anti-I-A–treated mice. 10^4 cloned T cells were cocultured with various doses of 2,000 rad irradiated APC and, when appropriate, KLH, in a final volume of 0.2 ml of E/R in flat-bottom, 96-well microtiter plates. [^{3}H] Thymidine incorporation was assessed on day 2 or 3 as described above for MLC. APC were light-density cells collected from a discontinuous BSA gradient consisting of a 35% and an 11% layer, spun at 25,000 g. Cells from the interface between the layers were washed twice in E/R without FCS. This enrichment for light-density cells is specifically necessary to measure thymic APC function (14).

Results

Lyt-2$^-$,L3T4$^+$ T Cells Are Not Present in Spleen or Thymus from Anti-I-A–treated Mice.   The key question to be addressed regarding the defects in anti-I-A–treated mice is whether the absence of T cell functions generally associated with class II recognition (18)^3 is due to the absence of a subpopulation of T cells, or to functional defects in the subpopulation of T cells responsible for class II recognition. This question may be answered by analyzing expression of the cell surface markers associated with T cells' specificity for the class I and class II MHC subregions. Expression of the L3T4 marker correlates with specificity for class II-MHC gene products (23–27). Lyt-2 expression, on the other hand, usually correlates with T cell specificity for class I-MHC gene products (21–23). We studied expression of the L3T4 and the Lyt-2 antigens on spleen cells and

thymocytes from 3-wk-old anti-I-A–treated mice, using two-color flow cytometric analysis. The results obtained with spleen cells using GK-1.5 (recognizing L3T4) and anti-Lyt-2 (Fig. 1) show, in both adult and 3-wk-old normal spleen cells (Fig. 1, *top* and *bottom*), distinct populations of Lyt-2⁻,L3T4⁺ cells, and Lyt-2⁺,L3T4⁻ cells. In contrast, the subset of Lyt-2⁻,L3T4⁺ cells is entirely absent in spleen cells from 3-wk-old anti-I-A–treated mice (Fig. 1, *middle*). In a series of five experiments, 6.2 ± 0.05% (mean ±SE) of normal spleen cells expressed the Lyt-2⁻,L3T4⁺ phenotype, while in spleen cells from anti-I-A–treated mice, only 0.9
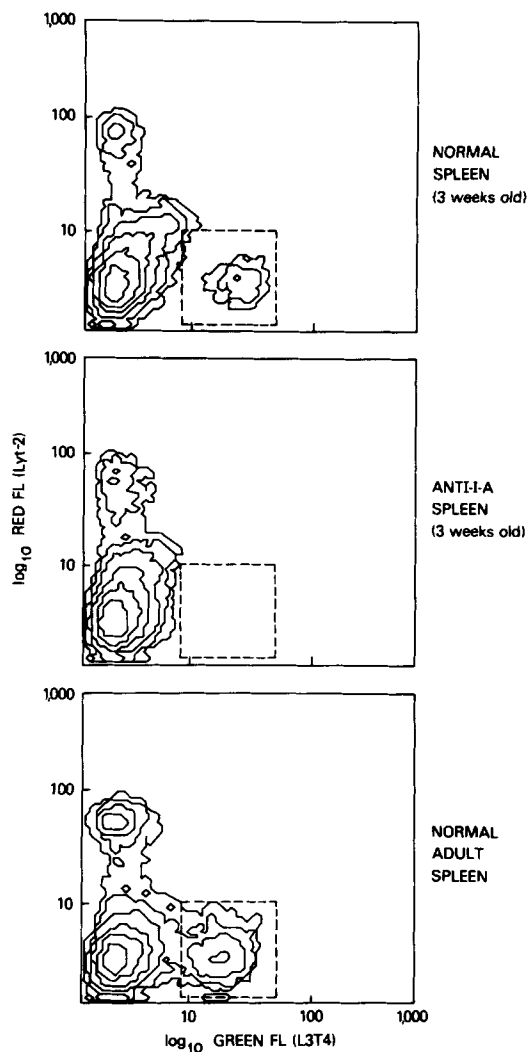

FIGURE 1. Two-color FACS analysis of Lyt-2 (red fluorescence, ordinate) and L3T4 (green fluorescence, abscissa) on spleen cells from control (*top*) and anti-I-Aᵇ–treated (*middle*) C57BL/6 mice. Spleen cells from 2–3 control or anti-I-A–treated mice were pooled and stained, first with FITC–anti-L3T4, then washed and stained with biotin-labeled anti-Lyt-2, followed by Texas Red–avidin. For comparison, a similar analysis of adult (10-wk-old) spleen cells is given (*bottom*). IL-2 production by spleen cells from these same mice is shown in Fig. 3.

± 0.2% Lyt-2⁻,L3T4⁺ cells were found. This suggests that the absence of allo–class II–specific and self–class II–restricted proliferative responses in spleen cells from anti-I-A–treated mice[3] is due to absence of the Lyt-2⁻,L3T4⁺ T cell subset responsible for these functions, rather than functional defects in such cells.

Since thymocytes from anti-I-A treated mice also exhibit inability to generate allo–class II–specific and self–class II–restricted proliferative responses,[3] it is likely that anti-I-A affects intrathymic T cell development as well. Two-color flow cytometry analysis of thymocytes supports this notion. In both adult (Fig. 2, bottom) and 3-wk-old normal thymocytes (Fig. 2, top), four subpopulations are detected after staining for L3T4 and Lyt-2: Lyt-2⁺,L3T4⁺ (representing the cortical type, immature T cells [30, 31]), Lyt-2⁺,L3T4⁻ (representing functional CTL precursors [31]); Lyt-2⁻,L3T4⁺ (representing functional class II–restricted
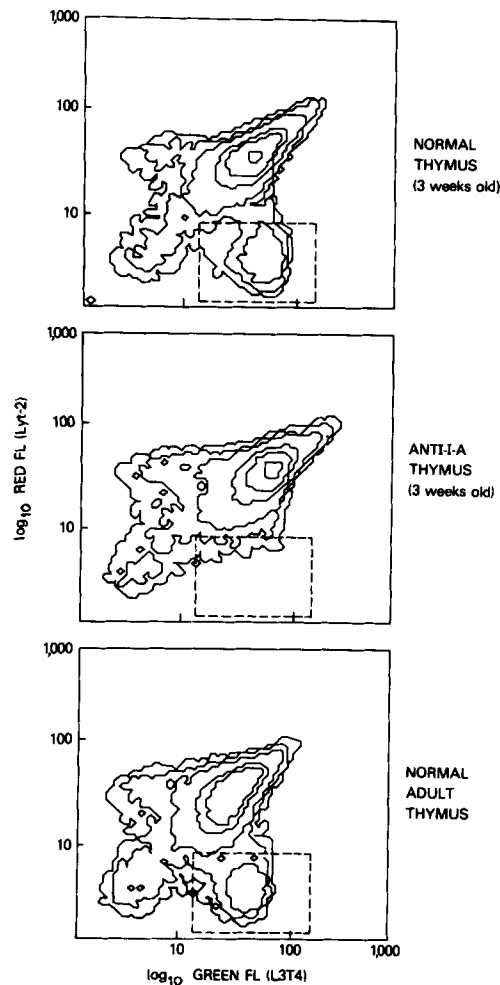

FIGURE 2. Two-color FACS analysis of Lyt-2 (red fluorescence, ordinate) and L3T4 (green fluorescence, abscissa) on thymocytes from control (top) and anti-I-Aᵇ–treated (middle) C57BL/6 mice. Legend as in Fig. 1. IL-2 production by thymocytes from these same mice is shown in Fig. 4.

IL-2-producing cells [30, 31]); and Lyt-2$^-$,L3T4$^-$, a small subpopulation including the dull Lyt-1$^+$,Lyt-2$^-$ (dLy1) precursor thymocyte (45). A striking difference emerged when thymocytes from 3-wk-old anti-I-A–treated mice were tested: the subpopulation of Lyt-2$^-$, L3T4$^+$ T cells is completely absent (Fig. 2, *middle*), with no apparent change in the other subpopulations. In a series of five experiments, 9.8 ± 0.01% Lyt-2$^-$,L3T4$^+$ cells are detected in normal thymus, while in anti-I-A–treated mice, only 1.7 ± 0.02% of these cells was present. Thus, anti-I-A treatment selectively affected development of the Lyt-2$^-$,L3T4$^+$ T cell subset.

Previous publications (23–27, 30) ascribed both antigen plus class II MHC–restricted or class II-alloantigen–specific proliferation and IL-2 production to Lyt-2$^-$,L3T4$^+$ cells. We therefore wished to determine whether the absence of the Lyt-2$^-$,L3T4$^+$ T cell subset in anti-I-A–treated mice was accompanied by lack of IL-2 production. Alternatively, anti-I-A treatment could have led to loss of the L3T4 marker, without loss of functions associated with this subset. Using a microculture system developed by others (41), IL-2 production in individual microcultures of normal and anti-I-A–treated spleen cells in response to stimulation by semiallogeneic stimulator cells was determined. Under such circumstances, most of the IL-2 produced is released by Lyt-2$^-$ cells, which respond preferentially to class II differences (30, 41, 42). Responder cells were pretreated with anti-Lyt-2 plus complement to remove cells that do not produce, but may use IL-2 (42). Recovery of spleen cells after such treatment was 70–80% from both normal and anti-I-A–treated spleen cells. As shown in Fig. 3, when the supernatants from individual microcultures were tested for IL-2, spleen cells from anti-I-A–treated mice exhibited an obvious decrease in the number of positive cultures. The frequency of IL-2-producing cells under these conditions was 1/189 for normal spleen cells, compared with 1/5,810 for spleen cells from anti-I-A–treated mice (mean of 10 separate experiments; see also Table I). The normal spleen cell values are within the range of those previously reported (41, 42, 46) for adult spleen cells. Similar results, i.e., a strong decrease in the frequency of IL-2-producing precursors in anti-I-A–treated mice, were observed when responder cells were not depleted of Lyt-2$^+$ cells (data not shown). We choose to present data with Lyt-2$^-$ responder cells only, because under such circumstances more accurate frequency determinations are obtained (42), presumably because Lyt-2$^+$ cells may use IL-2.

A similar analysis with thymocytes was found to be technically difficult: after treatment of normal thymocytes with anti-Lyt-2 plus complement, 6–10% of the cells could be recovered, while, with thymocytes from anti-I-A–treated mice, often only very few cells survived the treatment. Nonetheless, data representative of four interpretable experiments with Lyt-2$^-$ thymocytes are presented. A limiting-dilution analysis of the Lyt-2$^-$ thymocyte population of normal and anti-I-A–treated mice indicated (Fig. 4) that virtually no microcultures in the anti-I-A–treated group released detectable levels of IL-2; in three out of four experiments, a frequency of <1/10^4 was found (see also Table I). In contrast, even low numbers of normal thymocytes are, as reported earlier (30, 31), able to give rise to easily measurable levels of IL-2: for four experiments, an average frequency of 1/286 (see also Table I) of IL-2-producing precursors was observed. Comparable results were obtained with untreated responder thymocytes (data not
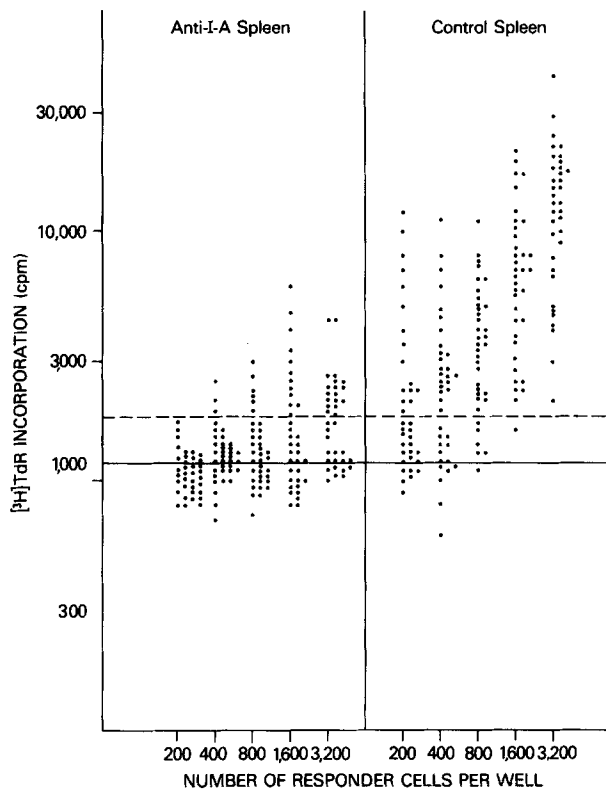
FIGURE 3.   Limiting-dilution analysis of IL-2 production in the spleen of control and anti-I-Aᵇ–treated C57BL/6 mice. Groups of 36 replicate microcultures were established of varying numbers of Lyt-2⁻ responder spleen cells, and 5 × 10⁴ T cell–depleted (B10 × B10.D2) nude peritoneal stimulator cells. On day 5, 100 μl of supernatants was harvested from each culture, and tested for ability to support growth of CTLL cells. Each dot represents IL-2 activity of an individual microwell. Positive wells were defined as those in which the activity exceeded the mean of control wells receiving no responder cells (solid line) by more than three SD of the mean (broken line). For frequency values, see Table I.

shown). Taken together, these data demonstrate that in vivo anti-I-A treatment results in a T cell function defect strictly confined to the Lyt-2⁻,L3T4⁺ subset. Such cells are markedly decreased in either thymus or spleen from anti-I-A–treated mice, and functions associated with this subset, i.e., class II-MHC–restricted T cell proliferation[3] and IL-2 production are strongly diminished, or absent.

Correlation of Defective Proliferative T Cell Response with Defective Thymic APC Function in Anti-I-A–treated Mice.   It was previously observed (18) that Ia antigen expression in thymuses from anti-I-A–treated mice was strongly diminished. It has also been shown (14, 15)[2] that one pathway of acquisition of T cell I-region recognition is through interaction in the thymus with a bone marrow–derived cell that functions as an APC. In view of the absence of class II–restricted T cell responses[3] and lack of the Lyt-2⁻,L3T4⁺ subset in anti-I-A–treated mice, it was of interest to analyze their thymic APC function. Splenic APC function was probed as well, because splenic Ia antigen expression is completely absent in

TABLE I

Frequency of IL-2-producing Cells among Lyt-2⁻ Thymocytes and Spleen Cells from Control and Anti-I-A–treated Mice

Exp.	Lyt-2⁻ responder cells	T cell–depleted stimulator cells	Frequency of IL-2-producing cells*
1‡	Control spleen	B10 × B10.D2 F₁	1/400
	Anti-I-A spleen		1/2,808
	Control thymus		1/356
	Anti-I-A thymus		1/9,535
2	Control spleen		1/89
	Anti-I-A spleen		1/3,081
	Control thymus		1/280
	Anti-I-A thymus		<1/10⁴
3	Control spleen		1/112
	Anti-I-A spleen		1/8,486
	Control thymus		1/153
	Anti-I-A thymus		<1/10⁴
4§	Control spleen	B6 × bm12 F₁	1/110
	Anti-I-A spleen		1/4,100
	Control thymus		1/349
	Anti-I-A thymus		<1/10⁴

* In all experiments a linear semilogarithmic relationship between the proportion of negative cultures and the dose of responder cells was observed.
‡ Experiment also shown in Figs. 3 and 4.
§ An experiment with an only allo-I-region difference was included to illustrate that most IL-2 production under the circumstances used is a reflection of I-region recognition.

these mice (17). The results in Fig. 5 show that spleen cells and thymic cells from anti-I-Aᵇ–treated B6 (H-2ᵇ) mice were defective in presenting KLH to an I-Aᵇ plus KLH–specific T cell clone. Similar results were obtained with two other I-Aᵇ plus KLH–specific T cell clones, and with two alloantigen-specific T cell clones that recognize I-Aᵇ antigens (data not shown). This strongly argues that anti-I-A–treated mice lack functional Ia-bearing APC in both the thymus and the spleen. The failure of cells from anti-I-A–treated mice to induce proliferation in several different clones suggest that the APC lack I-A, rather than expressing altered I-A molecules. Mixing experiments, using APC from both normal and anti-I-A–treated mice, excluded induction of suppressor cells as the cause for the abrogation of APC function in anti-I-A–treated mice (data not shown).⁴ The possible contribution of defective thymic and/or splenic APC function to the observed defect in class II–restricted T cell proliferative responses was also studied. B6 mice were treated for 1 wk with anti-I-Aᵇ, then left untreated for 2 wk, and subsequently tested for their ability to generate thymic and splenic proliferative responses in MLC. Fig. 6 shows a typical pattern of the effect of this treatment schedule. While splenic responses are still considerably suppressed in such mice, and, in fact, indistinguishable from mice treated continuously for

⁴ In vivo treatment of adult mice with anti-I-A antibodies: Disappearance of splenic antigen presenting cell function concomitant with modulation of splenic cell surface I-A and I-E antigens. 1985. A. M. Kruisbeek, J. A. Titus, D. A. Stephany, B. L. Gause, and D. L. Longo. *J. Immunol.* In press.
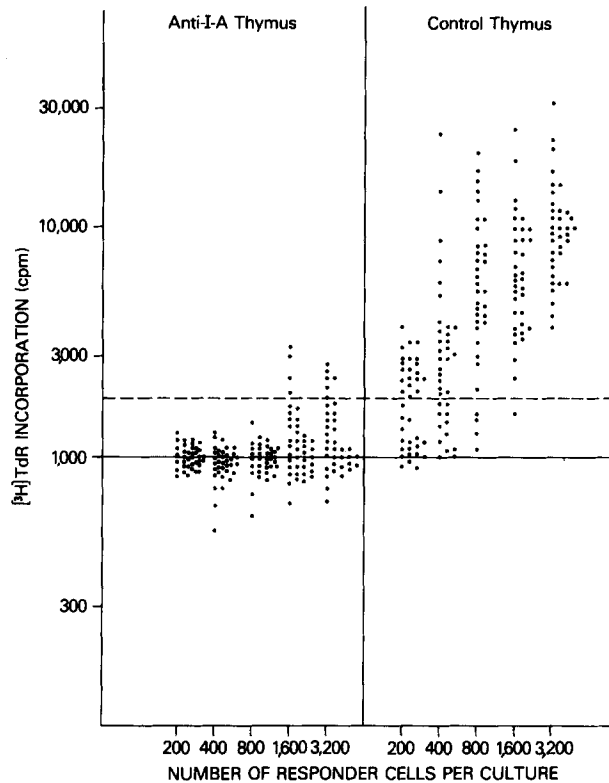
FIGURE 4. Limiting-dilution analysis of IL-2 production by thymocytes from control and anti-I-A^b–treated C57BL/6 mice. Conditions as described in legend to Fig. 3, except that thymocytes instead of spleen cells were used as responder cells.

3 wk with anti-I-A,[3] after 2 wk of recovery, thymocyte proliferative responses had returned to almost normal values. Note that 1 wk of treatment is sufficient to suppress both thymocyte and splenic MLC responses (data not shown). In addition, the number of Lyt-2⁻,L3T4⁺ T cells in the thymus returned to normal values 2 wk after anti-I-A treatment was terminated (data not shown). Thymic and splenic APC function were also tested 2 wk after termination of anti-I-A treatment. As shown in Fig. 7, thymic and splenic APC function from these mice is indistinguishable from that of untreated mice, even at low APC numbers. The mice used for the experiment in Fig. 7 are the same as those used for Fig. 6. Thus, an interesting picture emerges: when thymic APC function is reduced (Fig. 5), i.e., after 3 wk of continuous anti-Ia treatment, splenic and thymic class II–specific MLC responses,[3] as well as IL-2 production in response to an allogeneic stimulus (Figs. 3 and 4) are reduced, and Lyt-2⁻,L3T4⁺ cells are absent (Figs. 1 and 2.) However, when thymic APC function has become normal (i.e., 2 wk after treatment has been stopped), thymic MLC responses (Fig. 6) and Lyt-2⁻,L3T4⁺ cell numbers are returning to normal values, while splenic MLC responses are still diminished (Fig. 6). Return of splenic APC function is not correlated with return of splenic T cell responses. We interpret these findings as indicating that the generation of the T cell subset responsible for class II
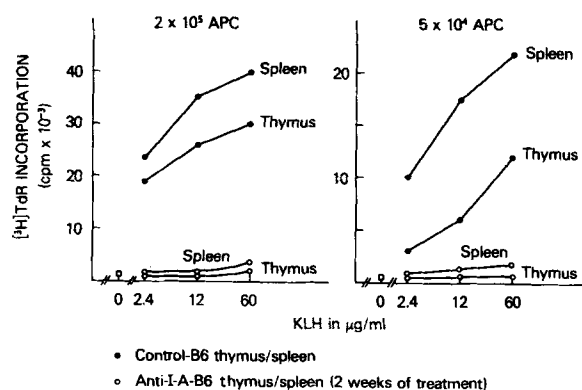
FIGURE 5. Splenic and thymic APC from B6 mice treated from birth with anti-I-A^b cannot present antigen (KLH) to KLH plus I-A^b-specific T cell clones. 10^4 cloned T cells from a KLH plus I-A^b-specific clone were cultured with the indicated doses of KLH, and either 2 × 10^5 or 5 × 10^4 2,000 rad irradiated APC, collected from the interphase of a BSA gradient consisting of a 35% and an 11% BSA layer. Cultures were labeled with 1 μCi [^{3}H]thymidine on day 2, and harvested on day 3. [^{3}H]Thymidine incorporation is expressed as the mean of triplicate cultures; standard deviation values were <14%. Control cpm values were 536 ± 67 for T cells alone, and ranged from 560 to 869 cpm for the various types and numbers of APC.

recognition depends on thymic, rather than splenic APC function, and that after anti-Ia treatment, new T cells capable of Ia recognition must be generated in the thymus before such cells can be detected in the periphery.

Discussion

Although the MHC phenotype of the nonlymphoid cells in the thymus has been implicated in the process that selects the MHC restriction specificities of T cells during development (1-15), the precise intrathymic events determining acquisition of MHC restriction specificities remain unresolved. Some of the most basic questions regarding the development of class I- and class II-specific T cells are: With which MHC-bearing nonlymphoid cells do developing T cells interact? Do cells destined to become class II-specific T cells interact with intrathymic elements different than those required for development of precursors of the class I-specific repertoire? Do intrathymic events determine the strong association between expression of L3T4 or Lyt-2 and, respectively, MHC subclass specificity for class II or class I? In the experiments reported here, an attempt is made to better understand development of MHC restriction specificities through interference with intrathymic expression of MHC antigens, specifically I-A antigens. This paper also describes the first experiments to have examined whether development of Lyt-2$^-$,L3T4$^+$ T cells can be separated from development of the Lyt-2$^+$,L3T4$^-$ T cell subset.

We present evidence that, in mice treated from birth with anti-I-A, the thymus and spleen do not contain Lyt-2$^-$,L3T4$^+$ T cells, as defined by dual-parameter FACS analysis. We also investigated whether absence of Lyt2$^-$,L3T4$^+$ T cells was accompanied by decreased IL-2 production, and found that anti-I-A-treated mice develop only very low levels of IL-2-producing precursors. Therefore, absence of the Lyt-2$^-$,L3T4$^+$ subset of T cells was defined both by marker
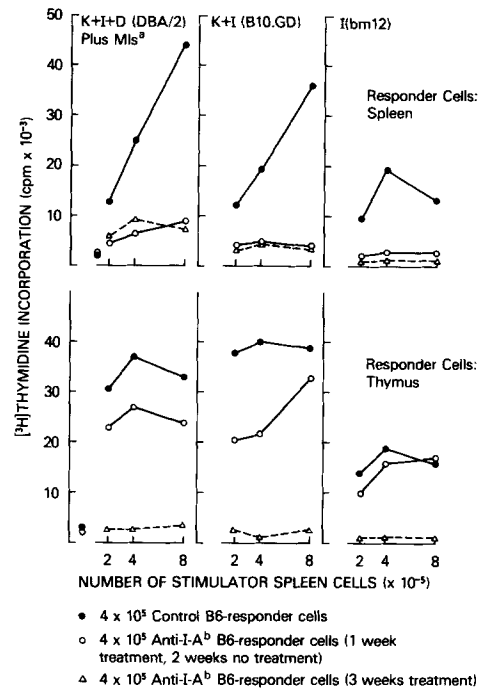
FIGURE 6.   Thymocyte proliferative responses to allogeneic stimulator cells recover before spleen cell responses in B6 mice treated neonatally with anti-I-A^b, after the treatment is stopped. 4 × 10^5 spleen cells or 6 × 10^5 thymocytes from control and anti-I-A–treated C57BL/6 mice were cultured with the indicated numbers of 2,000 rad irradiated allogeneic spleen cells, in a volume of 0.2 ml. Mice were either treated for 1 wk with anti-I-A^b, then left untreated for 2 wk before being tested (O), or treated continuously for 3 wk (Δ). 1 wk after treatment was terminated, thymocyte and spleen cell responses of mice from the same litter were still completely suppressed (data not shown). Spleen cell cultures were harvested on day 4; thymocyte cultures were harvested on day 5, each after a 16–20 h labeling period in the presence of 1 μCi of [^{3}H]thymidine. Control values in cultures containing stimulator cells only varied from 700 to 1,400 cpm. Responses to a class I alloantigen difference (H-2^{bm1} stimulator cells) or to M1s-disparate stimulator cells (D1.LP) behaved similarly, spleen cells being still almost entirely suppressed, and thymocytes exhibiting considerable recovery (data not shown).

analysis and by function. The presence of the lineage of Lyt-2⁺,L3T4⁻ cells was similarly defined both functionally (i.e., normal CTL precursors were present in these mice) (18), and through marker analysis: the dual-parameter FACS patterns demonstrate that the number of Lyt-2⁺,L3T4⁻ T cells in thymus and spleen is not affected by neonatal anti-I-A treatment.

Several points can be made regarding these findings. First, completion of the generation of the two major compartments of the T cell repertoire is clearly not a single event: development of the Lyt-2⁻,L3T4⁺ lineage can be selectively abrogated by a manipulation (i.e., neonatal anti-I-A treatment) that leaves the Lyt-2⁺,L3T4⁻ lineage unaffected. Our experiments do not distinguish whether the Lyt-2⁻,L3T4⁺ subset does not develop at all, or does develop, initially, but fails to expand to detectable levels. Perhaps it needs interaction with intrathymic I-A–bearing cells (see below) or factors produced by such cells as a signal to expand.
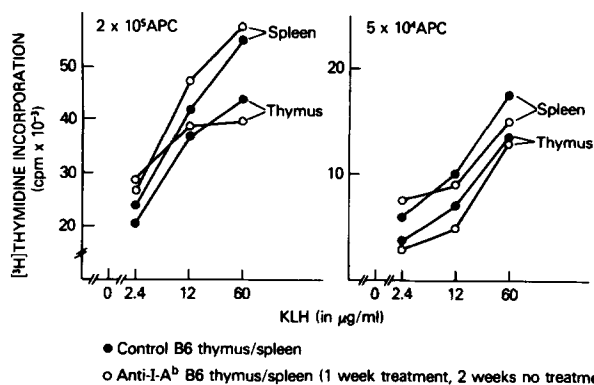
FIGURE 7. In B6 mice treated for 1 wk after birth with anti-I-A^b, APC function in spleen and thymus has returned to normal values 2 wk after the treatment is terminated. Conditions were as described in legend to Fig. 5. Thymus and spleen cell suspensions were from the same mice whose T cell proliferative responses are shown in Fig. 6. Control cpm values were 468 ± 71 for cloned T cells alone, and varied from 289 to 761 for the various types and numbers of APC.

Second, expression of the L3T4$^+$ marker is, in and of itself, not a feature dependent on intrathymic I-A–bearing cells, because normal levels (~80%) of cells staining positively for both L3T4 and Lyt-2 are found in the thymus of anti-I-A–treated mice, in accord with previous reports on the size of this "immature," cortical thymocyte population (30) in the normal thymus. There is currently no consensus on the relationship between the immature Lyt-2$^+$,L3T4$^+$ subset and the lineages of Lyt-2$^+$,L3T4$^-$ and Lyt-2$^-$,L3T4$^+$ cells (45, 47–49); it is not clear whether precursors for either one or both of these lineages are contained within the Lyt-2$^+$,L3T4$^+$ subset. Several studies (45, 49, 50) identified a dull Lyt-1$^+$, Lyt-2$^-$,L3T4$^-$ precursor cell of the Lyt-2$^+$,L3T4$^+$ subset, while recent[5] in vivo transfer studies demonstrated that this Lyt-1$^+$ dull precursor cell can give rise to all three other thymocyte subsets: Lyt-2$^+$,L3T4$^+$; Lyt-2$^-$,L3T4$^+$; and Lyt-2$^+$,L3T4$^-$. Our findings suggest that generation of the subsets of Lyt-2$^+$,L3T4$^+$ and Lyt-2$^+$,L3T4$^-$ cells is not dependent on I-A–bearing elements affected by anti-I-A treatment, while generation of the Lyt-2$^-$,L3T4$^+$ subset is. Models such as this one may aid in further dissecting the intrathymic differentiation pathways leading to the various T cell subsets.

Third, the question arises whether, in fact, all IL-2-producing T cells, regardless of their MHC subregion specificity, are affected by anti-I-A treatment. In our experiments, IL-2 production was induced by a full MHC difference or class II–alloantigen difference only, thus reflecting mainly activation of class II–specific Lyt-2$^-$ T cells, regardless of whether Lyt-2$^+$ T cells were present or absent in the responder cell population (30, 41, 42). Accordingly, no conclusion can be made about the capacity of Lyt-2$^+$ T cells to produce IL-2. Although the Lyt-2$^+$ subset, as defined by marker analysis, is apparently unaffected by anti-I-A treatment, additional experiments are required to determine whether the

[5] Early T lymphocytes: Differentiation in vivo of adult intrathymic precursor cells. B. J. Fowlkes, L. Edison, B. J. Mathieson, and T. M. Chused. Manuscript submitted for publication.

frequency of IL-2-producing Lyt-2⁺ cells is affected. To this end, stimulation with a class I–alloantigen difference only will be used (22, 23).

The absence of the lineage of Lyt-2⁻,L3T4⁺ T cells in the present model was correlated with absence of thymic APC function for class II–specific T cells. Thymic APC are bone marrow–derived (14, 16), and located in the medulla or at the cortico-medullary junction (16, 51). Most of the phenotypically and functionally "mature" thymocytes are located in the medulla (30, 31, 47, 52, 53). Taken together, these studies suggest that generation and/or expansion of the Lyt-2⁻,L3T4⁺ subset requires an interaction of an unknown thymocyte precursor cell with I-A-bearing elements, presumably APC, in the thymus medulla. Additional experiments are required to determine whether this interaction requires self–I-A recognition, as might be expected on the basis of experiments by others (54). Furthermore, thymic APC may have a nonspecific role in the expansion of thymocytes through release of factors such as IL-1. Finally, the question whether thymic APC have truly disappeared in anti-I-A–treated mice, or become non-functional through loss of Ia-antigens is currently investigated histologically, using a panel of mAb with which cortical and medullary thymic stromal cells can be distinguished on tissue sections (55).

Notwithstanding the correlation between lack of thymic APC and absence of the Lyt-2⁻,L3T4⁺ subset, a contribution of the lack of B cells in anti-I-A–treated mice (17) to the peripheral T cell defects may also be considered. Mice treated neonatally with anti-IgM (56–58) express altered Ig idiotype specificities in their T cell repertoire, in addition to lacking B cells. This suggests that idiotype-specific interactions between B cells and T cells contribute to the selection of T cell repertoire specificities. Such effects might also occur in anti-I-A–treated mice. Some differences between anti-IgM– and anti-I-A–treated mice, however, should be mentioned: (a) antigen-specific helper T cells are easily generated in anti-IgM–treated mice (57, 58), while anti-I-A–treated mice lack T cells of the phenotype commonly associated with helper cell function; (b) although anti-IgM–treated mice have impaired antigen-specific T cell proliferative responses, they express normal MLC reactivity (59), contrasting with the strongly impaired MLC reactivity in anti-I-A–treated mice³ (this report). It remains to be established whether the absence of the B cell compartment in anti-I-A–treated mice affects the T cell repertoire. We propose that a primary defect is an inability of the Lyt-2⁻,L3T4⁺ subset to develop or expand intrathymically in the absence of functional I-A-bearing APC.

Summary

In an effort to elucidate the role of intrathymic Ia-bearing antigen-presenting cells (APC) on the development of the class II–restricted T cell repertoire, we examined the effect of neonatal anti-I-A treatment on both intrathymic and splenic APC function; on the generation of Lyt-2⁻,L3T4⁺, Lyt-2⁺,L3T4⁻, and Lyt-2⁺,L3T4⁺ T cells; and on the development of class I– and class II–specific T cell functions. Both the thymus and the spleen are completely devoid of Lyt-2⁻,L3T4⁺ T cells in young mice treated from birth with anti-I-A, and also lack functions associated with this subset, i.e., alloantigen-specific interleukin 2 production (present report), allo–class II–specific and self–class II–restricted T cell

proliferative responses,[3] and helper cell function for the generation of cytotoxic T lymphocyte responses (18). Development of the $Lyt-2^+,L3T4^-$ subset proceeds undisturbed in these mice, in accord with the previously reported normal levels of cytotoxic T lymphocyte precursors (18). The thymus contains normal numbers of the immature cortical $Lyt-2^+,L3T4^+$ cells, indicating that acquisition of the L3T4 marker, in and of itself, is not influenced by anti-I-A treatment. This striking absence of the lineage of T cells responsible for class II–specific T cell functions is correlated with absence of thymic APC function for class II–restricted T cell clones. When anti-I-A–treated mice are allowed to recover from the antibody treatment, splenic and thymic APC function return to normal in 2–3 wk, and thymic $Lyt-2^-,L3T4^+$ T cell numbers and functions reappear before such cells are detectable in the spleen. Collectively, these findings suggest that development of the $Lyt-2^-,L3T4^+$ lineage of class II–specific T cells is entirely dependent on functional I-A-bearing APC cells in the thymus. In addition, the presence of normal levels of $Lyt-2^+,L3T4^-$ T cells argues that generation of the two major subsets of T cells (i.e., $Lyt-2^+,L3T4^-$ and $Lyt-2^-,L3T4^+$) occurs through separate events, involving unique sites of interactions between precursor T cells and nonlymphoid major histocompatibility complex–bearing thymus cells.

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