LONG-TERM HUMORAL UNRESPONSIVENESS IN VIVO, INDUCED BY TREATMENT WITH MONOCLONAL ANTIBODY AGAINST L3T4

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Immunotherapy in vivo with antibodies directed against T lymphocytes has been successfully used for immunosuppression in patients after organ transplantation, as well as in murine autoimmune disease models (1–4). The suppressive effect of such immunotherapy has been directly correlated with reductions in target cell numbers (5). Generation of mAbs with specificity for defined T cell subsets may allow a limited selectivity in this type of manipulation of the immune response. Although some monoclonal reagents have been extremely effective in eliminating T cells in vivo, the immunosuppressive effect generated has been transient and has lacked antigen specificity (6, 7).

Experiments in vitro and adoptive transfer of lymphocytes into irradiated recipients have shown that the generation of a humoral immune response against most antigens is T cell–dependent. Regulation and amplification of B cell function has been attributed to a T cell subset, which by cell surface phenotyping is characterized by the expression of a nonpolymorphic cell surface marker, the L3T4 molecule (8). This molecule is intimately involved in T cell activation; mAbs against L3T4 can specifically block Th cell effector functions in vitro. The presence of the L3T4 molecule is generally correlated with the specificity of mature T cells for MHC class II restriction elements (9, 10). Although there is a striking correlation between L3T4 expression and restriction specificity for MHC class II molecules, recent data (11) suggest that L3T4 may not represent a ligand-binding molecule for MHC class II molecules. The mechanism of action of the L3T4 molecule is unresolved.

It was the aim of this study to explore the function of $L3T4^+$ T lymphocytes in the initiation and differentiation of a humoral immune response in vivo. An additional objective was to analyze the immunocompetence of adult mice that had undergone helper cell depletion. A helper cell–deficient state may be associated with the induction of neonatal tolerance (12). During the early differentiation of the immune system the primary development of the T and B cell repertoire has been shown to be under genetic control (13, 14). Other mechanisms including antigen-driven differentiation might shape an individual's clonal profile (15, 16). The presence of soluble antigen in the periphery may

influence the relative frequencies of T cell and B cell clonotypes, which are subsequently expressed by the mature immune system. Elimination of L3T4+ Th cells in adult mice might make it possible to mimic neonatal conditions, and repeated injections of antigen during the early phase of the helper cell depletion and regeneration might help us study the influence of antigen on the ultimate composition of the T and B cell repertoire.

We established a model system in which the L3T4+ T cell subpopulation could be depleted in vivo by injection of anti-L3T4 mAb into adult mice. We monitored short-term, as well as long-term effects of the antibody treatment on the humoral immune responses against sperm whale myoglobin (SpWMb)[1] and KLH. After treatment with anti-L3T4 in vivo, adult mice did not develop primary humoral immune responsiveness against SpWMb or KLH. Such helper cell–depleted mice regained their ability to produce anti-KLH antibodies coincident with the recovery of the L3T4+ T cell subset, whereas we saw long lasting unresponsiveness against SpWMb which exceeded the apparent regeneration of the helper cell population. This long-term unresponsiveness to SpWMb could not be achieved in animals after memory induction. Thus, the immunosuppressive effect of anti-L3T4 therapy seemed to be determined in part by the preexisting T cell repertoire. The observed long-term unresponsiveness against SpWMb might suggest that the recovery of the L3T4 T cell population after helper cell depletion was a nonrandom process influenced by the presence of soluble antigen in the periphery.

Materials and Methods

Mice. BALB/c mice (6–8 wk old) were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. SpWMb was obtained from Sigma Chemical Co. (St. Louis, MO), and KLH was purchased from Calbiochem-Behring Corp., San Diego, CA.

Monoclonal Antibodies. The rat anti–mouse hybridoma GK1.5 was kindly provided by Dr. F. Fitch, University of Chicago. The antibody reacts with the L3T4 (CD4) molecules, and has been shown (8) to be of the IgG2b subclass. We obtained large amounts of purified antibody by passage of ascites over an affi-gel blue column (Bio-Rad Laboratories, Richmond, CA). The rat mAbs anti-Lyt-1 (hybridoma 53-7.2) and anti-Lyt-2 (hybridoma 53-6.7) have been described by Ledbetter et al. (17). Purified mouse mAb against rat κ chains (clone MAR 18.5, mouse IgG2a) was kindly provided by the laboratory of Dr. L. Herzenberg, Stanford University.

Fluorochrome Coupling. Biotin and fluorescein were purchased from Bio-Research, San Rafael, CA. Conjugated Texas Red avidin was obtained from Becton Dickinson & Co., Mountain View, CA. Biotinylation and fluorochrome conjugation have been described in detail (18, 19). Briefly, conjugations were performed in a carbonate/bicarbonate buffer at pH 9.3 for 4 h (biotin) or 2 h (fluorescein) at room temperature. Reagents were stored at 4°C with 0.1% azide. Immediately before use, they were deaggregated by spinning in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, CA) at 100,000 g for 10 minutes.

Staining Procedure. Lymph node cell suspensions of normal and treated mice were prepared in PBS containing 2% FCS and 0.1% sodium azide, and they were depleted of erythrocytes by gradient centrifugation on Ficoll-Hypaque. 5×10^5 cells were incubated in 25 μl of pretitered, conjugated antibody for 30 min on ice and were washed twice. For two-color analysis, green fluorescence was derived from directly FITC-conjugated reagents (anti-L3T4, anti-Lyt-2). In a second incubation, Texas Red–labeled avidin was added and bound to biotinylated first-step antibody (anti-Lyt-1). As controls for back-

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[1] *Abbreviation used in this paper:* SpWMb, sperm whale myoglobin.

ground fluorescence levels, we analyzed control cell preparations that were either un-stained or incubated with an isotype-matched irrelevant antibody. Flow microfluorometry analysis was performed on a modified FACS II system (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with logarithmic amplifiers. Data analysis by the VAX computer system has been previously described (20). Dead cells were excluded from analysis by the scatter gating method, and additionally by propidium iodine staining at a final concentration of 1 μg/ml. Data are presented on $\log_{10}$ scales of increasing green and red fluorescence intensity, and are shown as contour plots.

Immunization. Mice were immunized with 100 μg SpWMb or KLH intraperitoneally or received 100 μg of antigen emulsified in CFA. On day -1, 0, and 1, mice were injected intraperitoneally with purified anti-L3T4 mAb. Unless the experimental design required titrations of the antibody dose, the mice received 200 μg at each single dose. Sera were collected on days 6, 8, and 14 after immunization. 2 wk after the first antigen injection, the animals were boosted intraperitoneally with 100 μg aqueous antigen, and were challenged with a constant dose of 100 μg antigen at 2 wk intervals.

IL-2 Treatment. rIL-2 was used for reconstitution experiments. The recombinant product (No. 9C), as well as a buffer control, was kindly supplied by Cetus Corp., Emeryville, CA. In treatment experiments, each mouse received 200 μg anti-L3T4 on day -1, 0, and 1, followed by three daily injections of 5,000 U rIL-2 at day 1, 2, and 3. Control animals were injected with the control buffer to exclude nonspecific effects such as endotoxin contaminants.

LPS Stimulation In Vitro. Spleen cells of normal and treated animals were suspended in RPMI 1640 medium supplemented with 5% pretested FCS, 2 mM glutamine, and 10 mM HEPES. B cells were polyclonally stimulated in microcultures containing 2 $\times$ 10^5 spleen cells and 1% LPS in a total volume of 200 μl. Supernatants were harvested on day 3 and day 4, and secreted antibodies were measured in an ELISA system.

Determination of Antibody Titers. Antibody concentrations in sera and culture supernatants were determined in a solid-phase ELISA system (21). KLH or SpWMb was coated onto microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) by overnight incubation with PBS containing 100 μg/ml antigen. The remaining binding sites were saturated with a 2% BSA solution during a 3-h incubation at 37°C. Plates were washed extensively with PBS (0.05% Tween 20) and incubated with 50-μl aliquots of diluted serum for 2 h at room temperature. Antibodies fixed to the solid antigen phase were detected by using a pretitered goat anti–mouse Ig coupled with peroxidase. The developing reagents for total Igs were purchased from Tago Inc., Burlingame, CA. Goat anti-mouse antibodies specific for the isotypes IgG and IgM were obtained from Cappel Laboratories, Cochranville, PA. After incubation with the second stage antisera, plates were washed and developed using O-phenylenediamine dihydrochloride (1 mg/ml) and H_2O_2 as substrates in 0.1 M citric acid pH 4.5. 4 M H_2SO_4 was added to stop the reaction, and the OD480 was measured by an ELISA reader (Titertek Multiscan, Flow Laboratories, Inc., McLean, VA). Assays were standardized by using affinity purified hyperimmune sera against SpWMb or KLH.

Results

Elimination of L3T4$^+$ T Cells by Treatment In Vivo with anti-L3T4 mAb. Treatment in vivo with mAbs against the L3T4 molecule (CD4) has been used successfully to block certain T cell functions in mice (5). The mechanism(s) of action of the injected antibody is not clear. We used two-color FACS analysis to examine the effect of anti-L3T4 treatment in vivo on lymph node T cells. Directly conjugated reagents were used to detect the Lyt-1, the Lyt-2, and the L3T4 cell surface markers. Increasing amounts of purified anti-L3T4 mAb injected intraperitoneally caused a dose-dependent depletion of L3T4$^+$ T cells from the lymph nodes (Fig. 1). Amounts as little as 10 μg mAb given on each of 3 d were sufficient to eliminate 65% of the L3T4-expressing T cell subset (Table I). Injection of 50 μg mAb anti-L3T4 per day for 3 d depleted >90% of L3T4$^+$
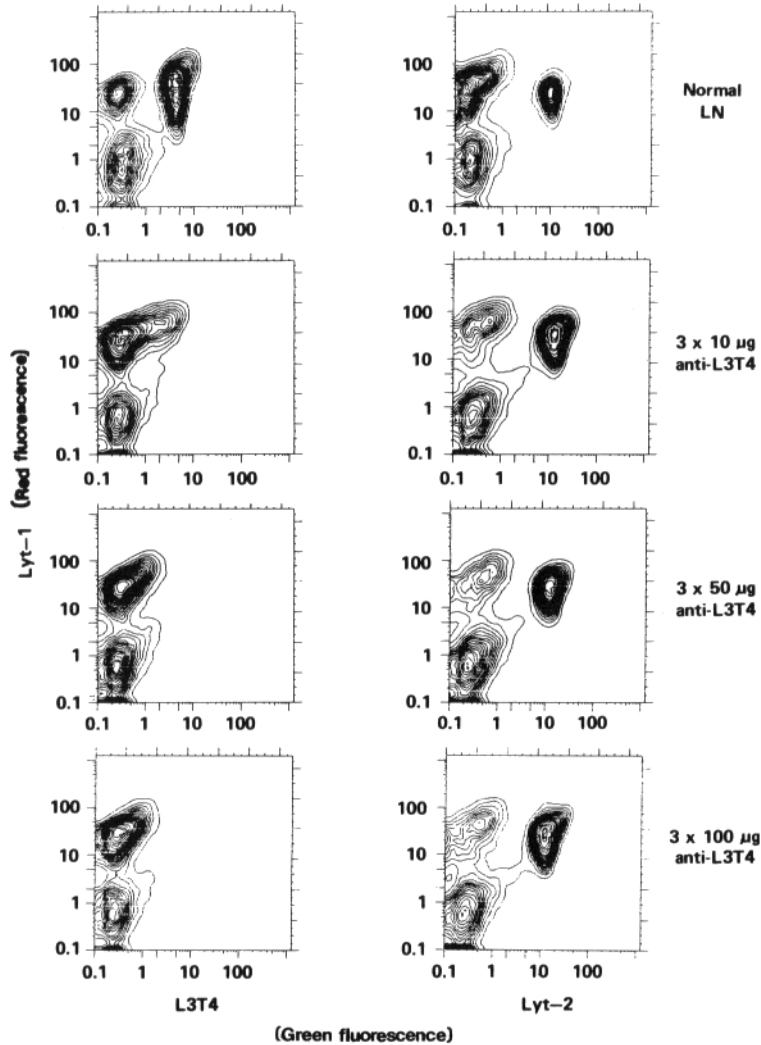
FIGURE 1. Two-color immunofluorescence analysis of lymph node cells after anti-L3T4 treatment in vivo. Doses of 10, 50, 100, and 200 μg anti-L3T4 mAb were injected into BALB/c mice on each of 3 consecutive days. Lymph nodes were harvested the day after termination of antibody treatment. Cells were stained with biotin coupled anti-Lyt-1 (hybridoma 53-72), and with fluorescence-labeled anti-L3T4 (hybridoma GK1.5) or anti-Lyt-2 (hybridoma 53-67). The biotinylated antibody bound to the cell surface was detected by Texas Red–labeled avidin as a second step reagent. Relative densities of the stained cell surface markers are represented as fluorescence intensity on $\log_{10}$ scales. Contour plots are used to express levels of green and red fluorescence. LN, lymph node cells.

T cells in the lymph nodes. A complete elimination of the L3T4$^+$ T cell subset was not achieved with antibody doses as high as 200 μg given on each of 3 consecutive days. After a total of 600 μg anti-L3T4, 5% of the remaining Lyt-1$^+$ T cells exhibited dull staining for the L3T4 cell surface marker by FACS analysis. Elimination of L3T4$^+$ T cells from the lymph nodes of treated animals was accompanied by a compensatory increase in cells of the Lyt-1$^+$,Lyt-2$^+$ phenotype (Table I). In addition, we found a small population of Lyt-1$^+$,Lyt-2$^-$

TABLE I

Dose-dependent Elimination of L3T4+ Lymph Node Cells After Treatment with Anti-L3T4 In
Vivo

Antibody treatment with anti-L3T4 (μg; for 3 d)	Total lymph node cells (× 10⁻⁷)	Percent lymph node cells expressing:						Ratio of L3T4/Lyt-2
		Lyt-1+	L3T4+	Lyt-2+	Lyt-1+, Lyt-2⁻	Rat Ig	Lyt-1+, Lyt-2⁻, L3T4⁻	
None	1.81	64.1	46.9	18.1	46.0	0	0	2.6
10	1.53	52.0	13.1	32.4	19.6	6.0	6.5	0.4
50	1.46	50.9	4.6	34.3	16.6	8.8	12.0	0.13
100	1.29	56.2	2.0	42.3	13.5	9.3	11.9	0.05
200	1.28	53.9	2.7	38.3	15.6	8.6	12.9	0.07

BALB/c mice were treated with the indicated doses of anti-L3T4 antibody; lymph node cells were
harvested and analyzed for the expression of cell surface markers as described in the legend to Fig.
1. Number of Lyt-1+,Lyt-2⁻,L3T4⁻ was calculated by the formula: Lyt-1+,Lyt-2⁻,L3T4⁻ = Lyt-1+ −
(Lyt-1+,Lyt-2+ + Lyt-1+,L3T4+).

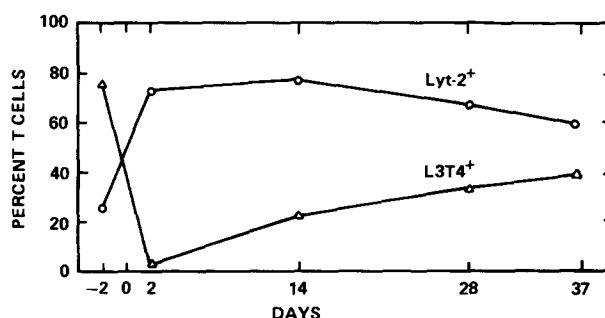FIGURE 2.   Reappearance of L3T4+ T lymphocytes in lymph nodes of anti-L3T4-treated
mice. BALB/c mice were injected intraperitoneally with 200 μg of anti-L3T4 mAb on each
of 3 consecutive days. Cervical, axillary, and inguinal lymph node cells of three mice were
pooled and cell surface phenotypes were analyzed by two-color FACS analysis before, and 2,
14, 28, and 37 days after treatment. Total numbers of harvested lymph node cells were 1.79
× 10⁷, 1.3 × 10⁷, 1.52 × 10⁷, 1.7 × 10⁷, and 1.72 × 10⁷, respectively, for the different days.
Lyt-1 was used as a global T cell marker. Lymph node cells staining for L3T4 or Lyt-2 are
expressed as percentages of total T lymphocytes.

lymph node cells in treated mice which exceeded the number of the remaining
L3T4+ cells. We analyzed these double negative cells (Lyt2⁻,L3T4⁻) for direct
binding of a mouse mAb against rat κ chains to examine the possibility that
residual rat anti-L3T4 antibody remained bound to certain L3T4+ cells. A
subpopulation of about 6–9% of lymph node cells reacted with the anti rat κ
chain antibody, suggesting that some L3T4+ cells remained that had bound rat
anti-mouse L3T4 antibodies. However, this subpopulation could not totally
account for the double negative cells, suggesting the emergence of a small
percentage of cells that had lost the expression of the L3T4 marker. Thus,
treatment in vivo with anti-L3T4 resulted in a depletion of most, but not all,
L3T4+ T cells from lymph nodes.

We analyzed lymph node cells of BALB/c mice for several weeks after the
termination of antibody treatment to study the persistence of the effects in vivo.
As shown in Fig. 2, mice treated with a total dose of 300 μg anti-L3T4 in three
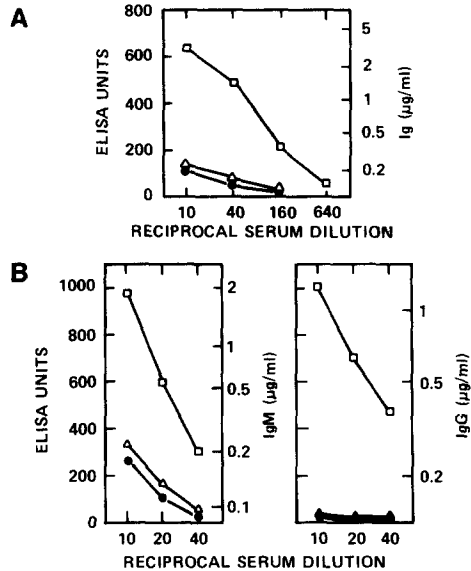divided doses had a long-term inversion of the L3T4/Lyt-2 ratio. For at least 4

FIGURE 3. Primary immune responses against soluble antigens in mice treated with anti-L3T4. (A) BALB/c mice were immunized with 100 μg aqueous SpWMb on day 0. Mice received either 200 μg of anti-L3T4 the day before, the day of, and the day after immunization (△), or were untreated (□). Sera before immunization (●) and collected 8 d after antigen injections were analyzed for SpWMb-specific antibodies. Results represent means of four mice in each group. (B) BALB/c mice were depleted of helper cells by injection of 200 μg anti-L3T4 over 3 d. On the second day they were immunized with 100 μg KLH. Titers of KLH-specific IgM and IgG in control animals (□) and antibody treated mice (△) were measured at day 8 after immunization. Each group consisted of four mice. Results are given as means.

wk, the majority of lymph node cells expressed the Lyt-2 marker. During the early posttreatment period a slow increase in L3T4⁺ cells was correlated with the disappearance of double negative Lyt-1⁺ lymph node cells. The repopulation of lymph nodes with L3T4⁺ T cells was dose-dependent. Mice treated with three doses of 10 μg anti-L3T4 regained 50% of the L3T4 subpopulations within 2 wk after the treatment (data not shown). Injection of higher doses caused an imbalance of peripheral T cell subsets for >4 wk.

Effect of Anti-L3T4 Treatment on the Primary Humoral Immune Response. Treatment in vivo with anti-L3T4 provided a reproducible animal model to study the effects of altered T cell composition on immune response. We analyzed the effect of anti-L3T4 injections on the humoral immune response against two soluble antigens, SpWMb and KLH. Immunization of normal untreated BALB/c mice with 100 μg soluble SpWMb induced a primary antibody response that was exclusively of the IgM subclass, and upon rechallenge, elicited a secondary IgG response (data not shown). Administration of SpWMb in CFA resulted in a significant production of IgM, and additionally, the emergence of IgG antibodies in the primary response. Immunization with KLH in soluble form without adjuvants elicited a primary IgG response in addition to IgM. We injected BALB/c mice intraperitoneally with 200 μg anti-L3T4 on 3 consecutive days (−1, 0, and +1). On day 0, the animals were immunized intraperitoneally with SpWMb or KLH. As shown in Fig. 3, treatment with antibody prevented a primary humoral immune response. No specific antibodies against SpWMb or

KLH were detected in sera of anti-L3T4-treated mice compared with untreated controls. However, mice treated with three doses of 10 μg anti-L3T4 had an almost normal antibody response to both antigens, although this dose of antibody significantly diminished the L3T4$^+$ T cell subpopulation (Table I). As determined in antibody titration experiments, 50 μg anti-L3T4 injected on each of 3 consecutive days was the minimal antibody dose that inhibited humoral immune responsiveness against SpWMb (data not shown).

Long-term Unresponsiveness of Animals after Treatment In Vivo with Anti-L3T4. BALB/c mice that had been treated with 3 × 200 μg anti-L3T4 at the time of primary immunization were subsequently boosted with soluble SpWMb or KLH at 2 wk intervals. To determine whether immunization with antigen at the time of the anti-L3T4 treatment was necessary for a specific blockade of the humoral immune response, two additional treatment groups were studied. Mice were treated with anti-L3T4 only, and immunizations were started either 2 or 4 wk later. None of the different experimental groups treated with anti-L3T4 developed a detectable IgG response against SpWMb, as shown in Fig. 4A. All of these groups of mice have been followed for ≥120 d. Mice that were immunized with SpWMb at the time of antibody injection and those that received anti-L3T4 treatment 28 d before immunization remained unresponsive, despite repeated challenge with SpWMb at 14 d intervals.

The antibody-induced elimination of L3T4$^+$ cells, however, could not induce similar long-term unresponsiveness against KLH (Fig. 4B). BALB/c mice that had been immunized with KLH 14 d after the anti-L3T4 treatment developed a primary IgM anti-KLH response, and subsequently, a secondary IgG response. Animals immunized with KLH 4 wk after anti-L3T4 injection had an almost normal antibody response to KLH.

Coadministration of KLH together with the anti-L3T4 antibody, however, decreased the ability of treated animals to develop a normal humoral immune response. Although originally unresponsive, such animals began to produce anti-KLH antibodies after the third antigenic challenge (4 wk after treatment). Serum levels of anti-KLH antibody in mice receiving coincident anti-L3T4 and KLH were lower than those of L3T4 depleted mice which were immunized with KLH 2 or 4 wk after antibody treatment (Fig. 4B).

Effect of Treatment In Vivo with Anti-L3T4 mAb on Secondary Immune Responses. Mice were primed with 100 μg SpWMb in CFA and rechallenged with 100 μg soluble SpWMb on day 14. Half of the mice were treated with divided doses of 200 μg anti-L3T4 mAb on the day before, the day of, and the day after rechallenge. Control mice, primed and rechallenged, increased their antibody titers against SpWMb from 1:640 to 1:10,240 (Fig. 5). Treatment of primed mice with anti-L3T4 mAb at the time of rechallenge prevented the induction of a secondary humoral response when measured on day 8 after rechallenge. This effect of anti-L3T4 mAb treatment on blocking secondary IgG antibody production, however, was transient. SpWMb-primed animals, treated with anti-L3T4, responded to the secondary antigenic challenge after an initial delay (Fig. 6). Just before the third SpWMb injection, the anti-L3T4-treated mice showed a significant, but diminished IgG antibody response against SpWMb. After the third immunization, antibody concentrations of treated mice reached the level that had been seen in the secondary response of control animals. The IgG

B

anti-L3T4	KLH [day]			
	0	14	28	42
▲	-	+	+	+	+
○	+	+	+	+	+
△	+	-	+	+	+
●	+	-	-	+	+

A

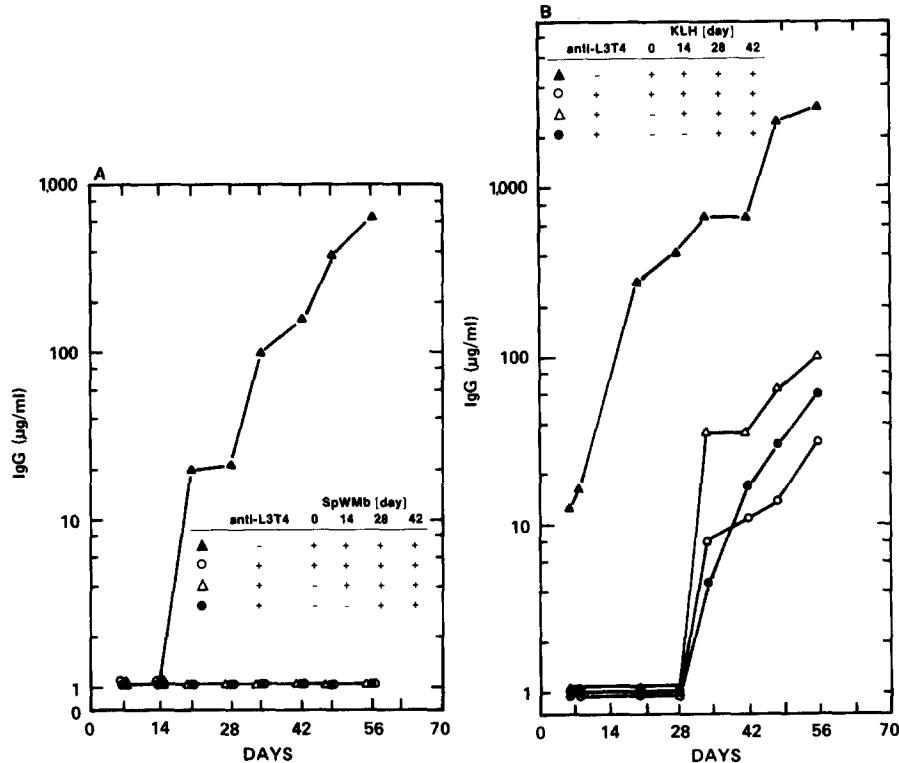| anti-L3T4	SpWMb [day]			
	0	14	28	42
▲	-	+	+	+	+
○	+	+	+	+	+
△	+	-	+	+	+
●	+	-	-	+	+

FIGURE 4. Long-term inhibition of humoral immunity against SpWMb in mice treated with anti-L3T4 in vivo. BALB/c mice were treated with anti-L3T4 (200 μg per day for 3 d) and were immunized with 100 μg soluble SpWMb at the time of treatment (O), and 2 wk (△), and 4 wk after (●) termination of the antibody treatment. One group did not receive antibody treatment (▲). Immunization was started on day 0. Each group of mice was rechallenged with 100 μg antigen every 14 d; serum concentrations of SpWMb IgG were monitored for 56 d. Results are expressed as means of four mice. (B) Recovery of humoral responsiveness against KLH in mice treated with anti-L3T4 in vivo. Mice were treated with anti-L3T4 and immunized as described in A, except that 100 μg KLH was used as the immunizing antigen instead of SpWMb. Results are given as mean serum concentrations of KLH-specific IgG antibodies.

production in the anti-L3T4-treated mice was, however, still diminished when compared with controls 5 wk after administration of the antibody. Additionally, anti-L3T4-treated primed animals characteristically showed a delayed responsiveness when challenged with antigen (Fig. 6).

Anti-L3T4-treated Mice Have a Normal B Cell Repertoire. To examine B cell repertoire in anti-L3T4-treated mice, we measured the LPS-induced production in vitro of anti-SpWMb specific antibodies. Spleen cells of treated animals were polyclonally stimulated with LPS 10 d after injection of a total dose of 600 μg anti-L3T4. Supernatants were harvested 3 d later and SpWMb-specific antibodies were measured in an ELISA system. LPS-stimulated splenic B cells of anti-L3T4-treated BALB/c mice produced as much IgM anti-SpWMb as did untreated controls (Fig. 7).

Effect of Exogenous rIL-2 in L3T4+ T Cell–Depleted Mice. The Lyt2−,L3T4+ T cell subset produces the lymphokine IL-2. We asked whether administration in vivo of exogenous rIL-2 could substitute for the greatly reduced number of
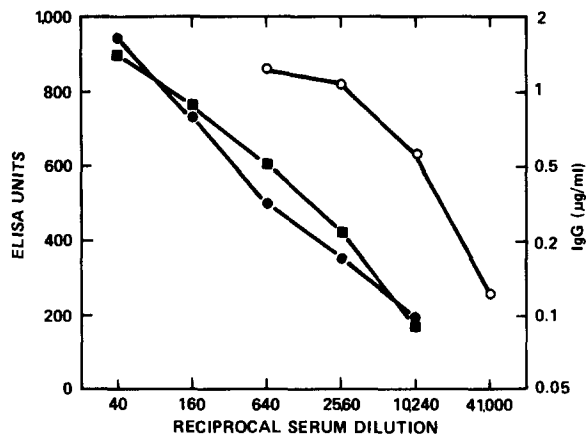
FIGURE 5. Effect of treatment with monoclonal anti-L3T4 on antibody production to SpWMb in primed animals. BALB/c mice were immunized with 100 μg SpWMb in CFA. 2 wk later, serum titers of SpWMb-specific IgG (■) were obtained and mice were challenged with 100 μg soluble SpWMb. Half of the mice were treated with a total dose of 600 μg anti-L3T4 given over 3 d (●), the other half remained untreated (○). Antibody responses to the antigen rechallenge measured on day 8 are shown as means of four mice.
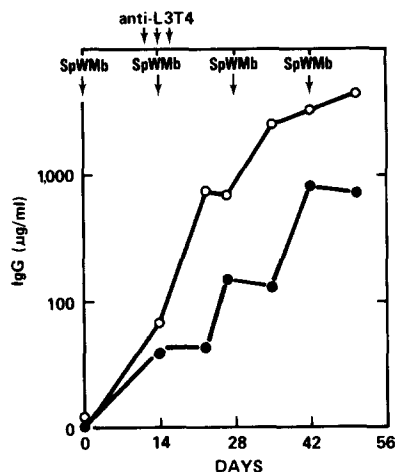

FIGURE 6. Long-term effect of anti-L3T4 treatment on the humoral responsiveness of primed animals. BALB/c mice that had been primed to SpWMb were treated as described in Fig. 5. Anti-L3T4 treated (●) and untreated (○) mice were boosted with 100 μg soluble SpWMb at 2 wk intervals. Their IgG responses to SpWMb were measured after each antigen injection. Each group consisted of four animals. Results are expressed as means.

L3T4+ cells in treated animals and reconstitute immune responsiveness in these animals. BALB/c mice were depleted of helper cells by injection of anti-L3T4, immunized with soluble KLH, and treated with three daily doses of 5,000 U rIL-2 for 3 d. We have previously shown that administration in vivo of this amount of rIL-2 can induce a polyclonal IgM response that is not dependent on the presence of antigen (22). The same result was observed in anti-L3T4-treated animals (Fig. 8, *left*), suggesting that: (*a*) L3T4+ T cells might not play a crucial role in this rIL-2-induced polyclonal IgM response; and that (*b*) the B cells that
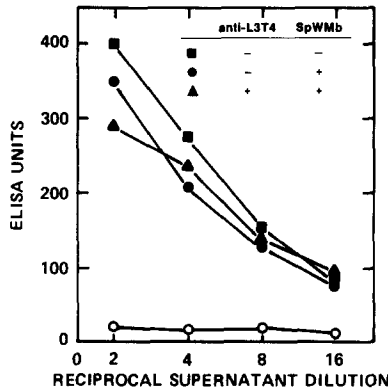
FIGURE 7.   B cell response in vitro of spleen cells from anti-L3T4 treated animals. BALB/c mice were immunized with 100 µg SpWMb; one group (▲) was additionally treated with 200 µg anti-L3T4 on days −1, 0, and 1; a second group (●) did not receive antibody treatment. A third group did not receive either anti-L3T4 or antigen (■). 10 d after treatment, 10⁶ splenocytes per ml were stimulated with 1% LPS. Culture supernatants were harvested after 3 d, and were analyzed for IgM antibodies to SpWMb.
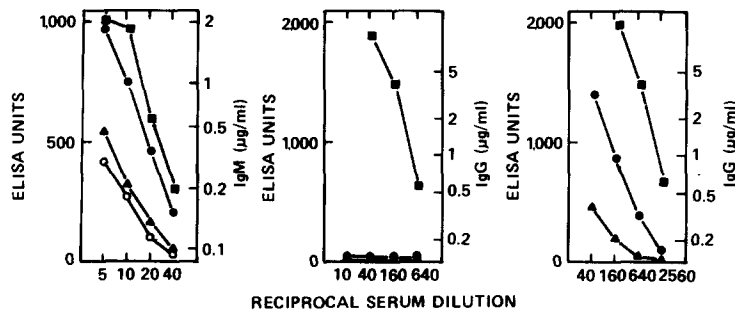

FIGURE 8.   Humoral responsiveness in mice treated with anti-L3T4 and reconstituted with rIL-2 in vivo. BALB/c mice were immunized with 100 µg soluble KLH. Mice remained either untreated (■) or were treated with anti-L3T4 in divided doses of 200 µg over 3 consecutive days (●, ▲). The antibody treated groups received either rIL-2 (●) the 3 d after antigen injection, or the buffer control (▲). rIL-2 was given in three daily doses of 5,000 U. Each group consisted of four mice. Mice were boosted with 100 µg KLH every 2 wk and sera were monitored for production of anti-KLH antibodies. The *left panel* shows IgM titers to KLH 8 d after primary immunization. IgG titers to KLH were measured 8 d after the second (*middle*) and 8 d after the third antigenic challenge (*right*). Results are given as means. (○) Normal mouse serum.

responded to rIL-2 had a normal repertoire, including IgM anti-SpWMb antibodies.

Although the anti-L3T4-treated, KLH-immunized mice developed IgM antibodies to KLH after rIL-2 administration, they did not produce a KLH-specific IgG response after a second antigenic challenge 2 wk later. Thus, administration of rIL-2 was not sufficient to induce memory in anti-L3T4-depleted, antigen-challenged animals, nor could it induce a class switch from IgM to IgG. The KLH-immunized mice treated with anti-L3T4 and rIL-2 were indistinguishable from the anti-L3T4-treated mice in the secondary response (Fig. 8, *middle*; however, they reacted differently after the third KLH challenge. As shown in Fig. 8, *right*, the humoral immune response (IgG anti-KLH) of rIL-2-treated mice was markedly enhanced compared with the response of animals that had

not received rIL-2, but administration of rIL-2 did not influence the delay (of about 4 wk) in the ability of anti-L3T4-treated mice to mount a humoral immune response against KLH. It is highly unlikely that the injected rIL-2 persisted in these animals for a period of 4 wk, suggesting that the effect of rIL-2 on the IgG production measured in the tertiary and quaternary humoral immune response had in some way been initiated at the time of the antibody treatment and the initial immunization.

Discussion

Experiments described in this paper were designed to investigate the functional importance of the L3T4$^+$ T cell subset in the initiation and differentiation in vivo of a humoral immune response against soluble antigens. Effective elimination of the L3T4$^+$ T cell population prevented the induction of a humoral immune response against SpWMb, as well as KLH. Abrogation of immune responses against BSA, OVA, sheep red blood cells, and KLH after the injection of anti-L3T4 have been reported (5, 23, 24). In the early posttreatment period, simple depletion of Th cells could account for the observed immunosuppression (Fig. 1 and Table I). Interestingly, we did not find a direct correlation between the reappearance of the L3T4$^+$ T cells and the restoration of the humoral immune responsiveness. Anti-L3T4 treated mice failed to respond to SpWMb, whereas a humoral immune response to KLH could be detected as early as 4 wk after anti-L3T4 treatment.

In addition to the striking reduction in numbers of L3T4$^+$ T cells, an impairment in APC function of treated animals early after the antibody treatment might have contributed to the inhibition of antibody production. L3T4 molecules have been shown to be expressed by rat macrophages (25), suggesting that administration in vivo of anti-L3T4 might block antibody formation through an effect on APC function. We saw a transient decrease in APC function after anti-L3T4 treatment, suggesting the L3T4 molecules are expressed on murine splenic APCs (unpublished observations). However, a single effective treatment in vivo with anti-L3T4 created a persistent inhibition of the humoral immune response for >4 wk, whereas splenic APC function of anti-L3T4-injected animals had returned to normal levels by 4 wk after antibody treatment. Additional experiments in vitro and in vivo showed that the B cell repertoire of treated mice was not affected (Figs. 7 and 8).

Both during titration studies on depletion and after the regeneration of the L3T4$^+$ T cell subset, a clear difference was observed between the response to KLH and SpWMb. A 40% reconstitution of the L3T4-expressing T cell population was sufficient to induce an antibody response against KLH, but could not initiate an antibody response against SpWMb. $\leq$90% depletion of L3T4 T cells affected SpWMb, but not anti-KLH responses. One possible explanation for this discrepancy, assuming that the elimination, as well as the regeneration of the T cell repertoire is random, would be that there are significantly less precursors of SpWMb-reactive T cells compared with KLH-reactive T cells. We have determined precursor frequencies of T cells that proliferate in response to SpWMb. Lymph node cells of a naive DBA/2 mouse contain about 1 in 45,000 anti-SpWMb proliferative precursors. Priming with soluble antigen increased this frequency to ~1 in 12,000 (22). Frequencies of 1 in 6,000 KLH-specific helper

cells were reported after immunization of mice with KLH in CFA (26). Thus, the preexisting T cell repertoire might determine the effects of anti-L3T4 on specific immune responses. Further evidence that clonal depletion may be one mechanism of immunosuppression after anti-L3T4 treatment in vivo comes from experiments done in primed animals. L3T4 T cell depletion only transiently affected antibody production in such mice (Fig. 6). In contrast to primary responses, as few as 20% recovered helper cells (L3T4$^+$) were sufficient in primed animals to allow IgG production against SpWMb. The preservation of memory antibody responses raises the interesting possibility that memory T cells may be selectively protected from the effect of in vivo of anti-L3T4.

It is possible that the elimination of L3T4$^+$ helper cells in the adult mouse could mimic the neonatal state and provide a short "window," allowing specific tolerance induction to an antigen present during the early phase of helper T cell regeneration. To test for active suppression, we adoptively transferred lymph node cells from such treated animals into irradiated recipients. In preliminary experiments, we failed to show antigen-specific suppressor cells (data not shown). To evaluate the potential importance of coincident administration of antigen on presumed tolerance induction, we compared humoral responses in animals that received SpWMb together with the anti-L3T4 injection, with those immunized with SpWMb 2 or 4 wk after the anti-L3T4 treatment (Fig. 4A). The finding that the mice that were first challenged with SpWMb 4 wk after antibody treatment did not develop a humoral immune response against SpWMb might argue against an antigen-induced unresponsiveness. However, additional experiments are necessary to analyze mechanisms underlying the long-term unresponsiveness after treatment in vivo with anti-L3T4.

An altered composition of the L3T4$^+$ population during the regeneration phase after antibody-mediated helper cell depletion might significantly contribute to the immunosuppressive effect. Evidence for a nonrandom elimination of L3T4$^+$ T cells and/or a nonrandom recovery of these T cells comes from several experimental observations. High doses of anti-L3T4 mAb, and thus elimination of 95% of L3T4$^+$ T cells, were necessary to inhibit anti KLH antibody production. However, $\geq$40% recovery of the L3T4$^+$ T cells was required to restore the response to KLH. Mice primed to SpWMb before anti-L3T4 treatment retained memory and could induce an anti-SpWMb IgG antibody response (Fig. 6). Further evidence for functional heterogeneity of the recovering L3T4 population was suggested by experiments in which Th cell–depleted mice were reconstituted with rIL-2. Mice treated with anti-L3T4 and rIL-2 generated significantly higher titers of specific antibodies after the third challenge (Fig. 8). The possibility that rIL-2-induced a more rapid recovery of T cells in the rIL-2-treated mice was excluded by two-color FACS analysis (data not shown). The finding of a delayed but markedly improved humoral immune response in rIL-2-injected mice suggested that rIL-2 might have expanded an amplifier T cell subset which potentiated the helper cell function for antibody production once helper cells reappeared. Our data provide evidence that humoral immunocompetence during and after T cell depletion by anti-L3T4 treatment is regulated by the representation of antigen-specific precursor cells within the T cell pool, as well as by additional immunoregulatory events that modulate humoral immune responsiveness. Further experiments are necessary to determine the exact nature

of different T cells involved in the reconstitution of a functional T cell compartment.

Summary

mAbs directed against the L3T4 molecule administered in vivo caused a severe and long lasting helper cell depletion in mice. Regeneration of the L3T4$^+$ subpopulation occurred gradually (2–3 mo) after a single antibody treatment. Experiments were designed to examine the humoral immunocompetence of such anti-L3T4-treated animals during and after regeneration of the L3T4$^+$ T cell subset. The animals were injected with anti-L3T4, immunized with soluble antigen, and challenged with antigen every 2 wk. Antibody responses to two antigens, sperm whale myoglobin (SpWMb) and KLH, which differ with regard to their immunogenicity, were compared. The lack of humoral immune responsiveness to either of these two antigens shortly after anti-L3T4 treatment was probably due to clonal depletion. The anti-L3T4-induced immunosuppressive effect on antibody production seemed to be determined in part by the preexisting T cell repertoire, as was suggested by the recovery of responsiveness to the highly immunogenic antigen KLH and the transient inhibitory effect of anti-L3T4 treatment in primed animals. The regenerating L3T4$^+$ T cell subpopulation was relatively incompetent in initiating B cell responses. More than 40% of the L3T4$^+$ T cell compartment had to recover to provide help for the production of anti-KLH antibodies, whereas elimination of 90% of the L3T4$^+$ helper cells did not inhibit a primary anti-KLH response. Evidence for a heterogeneous composition of the L3T4$^+$ subset came from experiments using rIL-2 in vivo. The addition of rIL-2 during early helper cell depletion improved the recovery of the humoral responsiveness without apparently affecting the kinetics of the regeneration of L3T4$^+$ T cells. Interestingly, humoral unresponsiveness to the weakly immunogenic antigen SpWMb persisted for at least 120 d. This long lasting unresponsiveness could not be explained by clonal depletion, and suggested as one possibility that the presence of antigen during regeneration of the L3T4$^+$ helper cell population may have influenced the ultimate T cell repertoire.

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