TWO STAGES OF B-CELL MEMORY DEVELOPMENT WITH DIFFERENT T-CELL REQUIREMENTS*

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Different approaches to determining the role T cells play in the development of B-cell memory have yielded apparently conflicting answers. Studies with T-deficient mice (adult thymectomized and reconstituted by bone marrow cells [ATX-BM],[1] nu/nu) have established that the antigen-induced appearance of memory B lymphocytes which give rise to IgG antibody-forming cells (AFC) requires the help of few (if any) mature T lymphocytes (1–3). These data appear inconsistent with studies which show that the affinity of the antibody produced by ATX-BM mice reconstituted with graded numbers of T cells increases with increasing T-cell dose (4). Since the avidity of antibody produced reflects the avidity of the antigen-binding receptors on the memory B cells (5), the T dependence of high avidity memory suggests that mature T cells are required for the antigen driven development or expansion of high avidity memory cells. This conflict is easily resolved if (as we will show in this publication) B-cell memory development proceeds in two stages: (*a*) the T-independent induction of a pool of B memory cells, and (*b*) the subsequent T-dependent evolution of high affinity memory.

The major problem in defining these two stages in memory development in nu/nu or ATX-BM mice is the separation of the direct effects of T depletion on avidity maturation from the possible indirect effects due to the overall reduction of T-dependent antibody synthesis. In our studies, we avoid this difficulty by using a new type of T-deficient mouse (allotype suppressed). These mice lack helper T (Th) for one population of memory B cells responsible for a small part of the total IgG antibody response in normal mice. They have normal Th for all other B-memory cell populations (6). With these mice, we can evaluate the effects of Th depletion on memory development in an environment where the bulk of the antibody response is proceeding normally, and thus where, in the same animal, memory development in a B-cell population lacking Th may be directly compared with memory development in B cells which have adequate Th.

The evidence for selective Th depletion in allotype-suppressed mice may be briefly summarized as follows: SJL × BALB/c hybrid mice used for these studies are Ig^b/Ig^a

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[1] *Abbreviations used in this paper*: AFC, antibody-forming cells, ATX-BM, adult thymectomized and reconstituted by bone marrow cells; D'PBS, Dulbecco's phosphate-buffered saline; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; Th, helper T cells; Ts, suppressor T cells.

immunoglobulin allotype heterozygotes. Because of allelic exclusion, these mice have two populations of IgG_{2a} memory B cells, one of which gives rise to AFC producing Ig-1b allotype and the other which gives rise to Ig-1a allotype AFC (7, 8). Under the priming conditions used, each of these populations accounts for approximately 10% of the total IgG secondary response (7, 8).

The Ig-1b memory B cells, which carry surface Ig-1b determinants[2] require help from specific Th (Ig-1b Th) in these hybrids. Th from carrier-primed congenic Ig^a/Ig^a mice help Ig-1a or IgG_1 memory B cells from the heterozygotes but do not help Ig-1b memory cells in an adoptive secondary response. Ig-1b memory expression occurs only if the heterozygous Ig^b/Ig^a B cells are supplemented with carrier-primed T cells from Ig^b/Ig^a nonsuppressed donors, i.e., with a source of Ig-1b Th (6).

Allotype-suppressed mice are SJL × BALB/c hybrids (Ig^b/Ig^a) exposed perinatally to antibody reactive with Ig-1b. These mice have a population of suppressor T cells which completely and specifically suppress Ig-1b production in situ and also in adoptive transfer with cells from nonsuppressed donors (7–9).

Carrier-primed allotype-suppressed mice do not have detectable Ig-1b Th activity. Their splenic T cells do not help Ig-1b responses in the adoptive secondary assay. (Suppressor T cells, which could mask Ig-1b memory expression, were killed with specific antisera or removed by size separation before transfer.) Th activity for Ig-1a and for other IgG classes, however, is normal in the suppressed mice (6).

In the studies presented in this publication, we use these Ig-1b Th-deficient allotype-suppressed mice to examine the role of helper T cells in the development of B-cell memory. We confirm our previous finding that the number of Ig-1b-bearing cells is the same in suppressed and nonsuppressed mice which originally suggested that Ig-1b memory development occurs in suppressed mice (10). (Ig-1b-bearing cells carry virtually all of the Ig-1b memory in spleen and do not carry memory for Ig-1a or other IgG classes [reference 7 and footnote 2].) We also show by adoptive transfer of B-cell populations from hapten-primed suppressed and nonsuppressed mice, that priming of Ig-1b memory B cells occurs normally in the absence of Ig-1b Th.

Continued Ig-1b memory development, however, is specifically impaired in Ig-1b Th-depleted mice. We show here that in primed mice boosted repeatedly with the priming antigen, the average avidity of most of the IgG memory cells increases over 100-fold but the avidity of the Ig-1b memory cells remains the same as after priming. Thus we conclude that Th which help Ig-1b memory cells are not needed for priming but are required for further avidity maturation.

Materials and Methods

Mice. SJL/JHz × BALB/cNHz hybrids (Ig^b/Ig^a) and BALB/cNHz mice were used in experiments reported here.

Media. For cell transfers: Dulbecco's phosphate-buffered saline (D'PBS), pH 7.5 (11), supplemented with 5% heat-inactivated fetal calf serum (FCS). For estimation of plaque-forming cells (PFC): Spleen cells to be tested were suspended in modified Eagle's medium (MEM) with Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.).

Antigens. Keyhole limpet hemocyanin (KLH) obtained from Pacific Bio-Marine Supply Co., Venice, Calif., was dialyzed for 24 h against 0.15 M NaCl, and then centrifuged at 10,000 rpm to remove precipitated protein. The resulting supernate was then centrifuged at 36,000 rpm in a

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[2] Okumura, K., M. H. Julius, T. Tsu, L. A. Herzenberg, and L. A. Herzenberg. 1976. Demonstration that IgG memory is carried by IgG bearing cells. *Eur. J. Immunol.* In press.

Beckman SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 90 min to pellet the protein, and the pellet resuspended in PBS (0.01 M phosphate, pH 7.1, in 0.15 M NaCl).

2,4-dinitrophenyl $(DNP)_8$-KLH (per 10^5 mol wt) was prepared by reacting dinitrofluorobenzene in 40-fold molar excess with the carrier protein for 3 h at pH 8.2 in 0.5 M $NaHCO_3$ buffer (12). Free DNP was removed by extensively dialyzing the conjugates against PBS.

Recipients for Cell Transfer Experiments. BALB/c mice irradiated with 600 R 18–24 h before transfer were used as recipients. Since the donors of the transferred cells in these studies are SJL × BALB/c hybrids, the cell graft is incompatible with the recipient. However, at the cell dose and irradiation dose levels used for our experiments we have observed greater than 90% long-term graft survival in a large series of experiments comprising more than 1,000 recipients. The recipients may show some effect of the incompatibility, however, in that allotype production and antibody response are somewhat elevated in the first 2 wk after transfer when compared to transfers of hybrid cells into syngeneic hybrid donors.

Donors for Cell Transfer Experiments. Nonsuppressed donors, 3- to 12-mo old, for adoptive cell transfers were obtained from matings between SJL/J males and BALB/cNHz females. Suppressed donors were obtained from matings between SJL males and BALB/c females immunized against the paternal Ig-1b allotype (13). Suppressed mice were 6- to 12-mo old when used, and had no detectable serum Ig-1b at time of sacrifice.

Priming. Nonsuppressed (SJL × BALB/c)F_1 mice received 100 μg DNP-KLH on alum plus 2 × 10^9 heat-killed *Bordetella pertussis* at least 6 wk before use as donors in adoptive transfer. Spleen cells from these mice were used as a combined source of hapten-primed B cells (memory B cells) and carrier-primed T cells or (after depletion of T cells as described below) as a source of hapten-primed B cells. KLH (carrier)-primed mice received 100 μg KLH on alum plus 2 × 10^9 *B. pertussis* 7 days before use as donors in adoptive transfer.

Boosting. For the avidity maturation experiment, DNP-KLH mice primed as described above received three injections of 10 μg DNP-KLH intravenously in saline at 6, 12, and 18 wk after priming. T cells were depleted (see below) from the spleen cell suspension obtained from the primed and boosted mice before testing in adoptive transfer.

Depletion of T Cells from Spleen Cell Suspensions. T cells were depleted form spleen cell suspensions by killing with anti-Thy-1 plus complement (C) in a "two-step" killing protocol. Cells were incubated with anti-Thy-1.2 (AKR anti-C3H) diluted 1:10 to 1:15 for 30 min at 37°C, centrifuged to pellet the cells, resuspended and incubated with guinea pig C, and diluted 1:10 for 30 min at 37°C. After the second step, cells were resuspended and washed once before transfer.

In some experiments a rabbit anti-mouse brain antiserum made specific for T cells or a congenic anti-Thy-1 serum (kindly supplied by Dr. E. A. Boyse and Dr. F. Shen at Sloan-Kettering Institute for Cancer Research, New York) was substituted for the noncongenic anti-Thy-1 generally used.

Adoptive Secondary Response to DNP-KLH. Donor cells were injected intravenously (i.v.) into BALB/c recipients irradiated (600 R) 18–24 h before transfer. Cell doses indicated in the text are equal to the number of viable cells transferred.

Recipients were challenged i.v. with 10 μg aqueous DNP-KLH immediately after transfer. 7 days later, recipients were killed, their spleens removed, and DNP-PFC estimated as described below. Since very little (<30%) variability in spleen size, cell yield, or response is usually found between individuals in the same group, three to five recipients were used per group and their spleen cells pooled before plaquing.

To assay for memory B cell, spleen cells from DNP-KLH-primed donors, or B cells (T-depleted spleen cells) from these donors plus a source of carrier-primed T cells were transferred to recipients. To assay for suppressor T cells, the test cell suspension was transferred together with DNP-KLH-primed spleen from nonsuppressed mice or with a mixture of DNP-KLH-primed B cells and carrier-primed T cells from nonsuppressed mice.

Estimation of DNP-PFC in Recipient Spleen. The PFC assay described by Cunningham and Szenberg (14) was used with 2,4,6-trinitrophenyl (TNP) sheep red cells (15).

The number of indirect PFC was determined by subtracting direct PFC per 10^6 (no developing antiserum) from the total PFC per 10^6 found when developing antiserum was included in the chamber.[2] In general, the direct PFC response was less than 2% of the total IgG response.

Avidity of DNP-PFC. Avidity was estimated by including ϵ-DNP-lysine in the plaquing chambers at 10-fold concentration steps from 10^{-10} to 10^{-5} M as previously described (16).

Developing Antisera. Total IgG PFC were developed with a pooled rabbit antiserum reactive with the Fc of all IgG classes, as well as with the Fab. The antisera detecting Ig-1a was made in a goat against the myeloma 5563 and rendered specific by absorbing with myeloma proteins of every other class and with the 50% saturated ammonium sulfate precipitate of C57BL serum in each case conjugated to Sepharose 4B. The starting serum for the anti-Ig-1b was produced by immunizing a rabbit with C57BL/6 (Ig^b) IgG (17). (This serum was kindly supplied by Dr. John Coe, Rocky Mountain National Laboratory, Denver, Colo.) This was absorbed by sequentially passing through columns containing BALB/c-Ig, Ig-1a (RPC-5 myeloma protein), and Ig-4b (MOPC-245 "T" myeloma protein) coupled to Sepharose 4B. All sera were tested for specificity by radioimmune assays and plaque development.

Immunofluorescent Staining of Ig-1b-Bearing Cells. For the staining of Ig-1b-bearing cells, the above anti-Ig-1b antiserum was specifically purified by absorption on Sepharose to which Ig^b (C57BL/10) immunoglobulins were covalently bound and eluting with glycine-HCl (0.1 M, pH 2.4) and immediate neutralization, and concentration to 1.5 mg/ml by dialyzing at reduced pressure. Immediately before staining, the antiserum was centrifuged for 1 h at 100,000 *g* to remove any aggregates.

Staining of NH_4Cl-treated (to lyse red cells) spleen cells was done as previously described (references 5, 18, and 19; and footnote 2). Briefly, 10 million pelleted spleen cells were resuspended in 0.1 ml of the anti-Ig-1b, diluted 1:10. After 30 min at 4°C the cells were underlayed with heat-inactivated FCS, centrifuged, and then washed once with D'PBS with 5% FCS. They were then resuspended in 0.1 ml of appropriately diluted fluorescein-conjugated goat anti-rabbit immunoglobulin. After 30 min at 4°C they were underlayed with FCS and washed as after the first step.

Counting of Fluorescent Ig-1b-Bearing Cells. Since less than 1% of spleen cells from heterozygous mice are Ig-1b-bearing cells, direct microscope counting of spleen cell smears is difficult and relatively inaccurate. Accuracy and speed were markedly increased by concentrating stained cells using the fluorescence-activated cell sorter (FACS) (20) (Becton Dickinson Electronics Laboratory, Mountain View, Calif.). The brightest 2, 5, and 10% of viable cells (21) were sorted into a well made by a small glass cylinder cemented to a microscope slide. After a sufficient number of cells were collected, the slide was centrifuged for 10 min in a Cytocentrifuge (Shandon Scientific Co. Ltd., London, England), the fluid aspirated from the well, and the cylinder broken off the slide. A drop of phosphate-buffered glycerine (pH 7.5) and a cover slip was then placed over the well area and the percentage of fluorescent cells counted by fluorescence microscopy. The percentage of Ig-1b-positive cells in the original suspension was calculated from this value by dividing by the enrichment factor in the FACS separation. Microscopy was with a Zeiss epi-illumination microscope (Carl Zeiss, Inc., New York) using standard excitation and barrier filtration (5).

Results

Development of Ig-1b Memory for DNP in Suppressed Animals. The data presented in Table I show that normal numbers of Ig-1b memory cells which can differentiate to Ig-1b DNP-PFC in an adoptive secondary response are present in spleens of chronically suppressed (SJL × BALB/c)F_1 mice primed 6 wk before transfer with DNP-KLH. These memory cells, however, are demonstrable only when the suppressor T cells (Ts), also present in spleens of suppressed animals, are first depleted by treatment of the spleen cell suspension with anti-Thy-1 and C and the memory B cells are supplemented with a source of cooperator T cells (Th) containing Th capable of facilitating the differentiation of Ig-1b memory cells (i.e., supplemented with [KLH] carrier-primed T from nonsuppressed animals). Without removal of Ts, primed suppressed spleen cells give rise to Ig-1a DNP-PFC (the allelically determined γG_{2a} antibody) and other Ig DNP-PFC but no detectable Ig-1b DNP-PFC. Anti-Thy-1-treated suppressed spleen transferred alone gives no IgG DNP-PFC response at all because the anti-Thy-1 also removes all Th. But supplementing with KLH (carrier)-primed nonsuppressed (SJL × BALB/c)F_1 spleen cells to provide Th gives rise to a substantial Ig-1b

TABLE I
B-Cell Memory Development in DNP-KLH-Primed Ig-1b Suppressed (SJL × BALB/c)F_1 Hybrids

DNP-KLH-primed spleen cells transferred (× 10^6)				Indirect PFC*		
Suppressed		Nonsuppressed				
B + Th + Ts	B‡	B + Th	B‡	Ig-1b	Ig-1a	Total IgG
10				<10	450	3,550
		10		530	640	6,180
	5			350	390	3,990
			5	450	530	4,970

* Indirect DNP-PFC/10^6 recipient spleen cells. Direct DNP-PFC (<50) subtracted.
‡ T cells in DNP-KLH-primed donor spleens were depleted by treatment with anti-Thy-1 plus C. T-depleted suspensions were supplemented with 10^7 KLH spleen cells (Th) from nonsuppressed (SJL × BALB/c)F_1 donors. For details of priming and transfer, see Materials and Methods section.

DNP-PFC response, as well as Ig-1a and total DNP-PFC. The number of Ig-1b DNP-PFC obtained differs only slightly from that obtained from nonsuppressed donors, indicating that normal numbers of Ig-1b memory B cells are present in primed suppressed spleen (see Table I).

The demonstration in Table I that the Ig-1a and Ig-1b responses from the suppressed donors are equal provides even better evidence on the same point. Since we have shown in numerous transfer experiments that these responses are always just about equal from normal donors, comparison of the Ig-1a and Ig-1b responses gives an internal control which is independent of differences in the magnitude of overall priming which can occur between groups of donors. Thus, the equality of the Ig-1a and Ig-1b responses strongly argues against even minimal interference with the amount of Ig-1b memory development in the suppressed animals.

Presence of Ig-1b-Bearing Cells in Spleen of Suppressed Animals. Table II shows that suppressed mice have normal numbers of Ig-1b-bearing lymphocytes (B cells). To determine the number of Ig-1b-bearing cells in spleens of suppressed and normal animals, spleen cell suspensions from individual animals were incubated first with an absorbed rabbit antiserum specific for Ig-1b and then with fluorescein-conjugated goat anti-rabbit immunoglobulin antibodies. Under the conditions used, only those cells which bind the first-step reagent (anti-Ig-1b) become visibly fluorescent with the fluorescence microscope. (The proof that the reagent is specific for Ig-1b is best demonstrated by separation data presented elsewhere,[2] which shows that Ig-1b-bearing cells isolated with this reagent, using the same staining conditions, carry only Ig-1b memory.) To facilitate counting, since less than 1% of the cells are Ig-1b positive, the suspensions were enriched 10-fold for fluorescent cells by FACS isolation of the brightest 10% of cells before making smears for microscope counting.

As the data in the table show, no significant differences exist between the number of Ig-1b-bearing cells found in suppressed and normal spleens. In both

TABLE II
Percentage of Cells with Surface Ig-1b in Normal and Suppressed Mice

SJL × BALB/c 8–10 mo	No. of animals	Serum Ig-1b	Ig-1b-bearing cells*
		mg/ml	%
Normal spleens	8	>0.5	0.54 ± 0.15
Suppressed spleens	7	<0.01	0.59 ± 0.11

* Spleen cell suspensions incubated with rabbit anti-Ig-1b and then with fluorescein-conjugated goat anti-rabbit. Cells were enriched by passage through FACS before counting by fluorescence microscopy. (See Materials and Methods.) Numbers are arithmetic mean and standard deviation.

cases, between 0.5 and 0.6% of the spleen cells are Ig-1b positive. This confirms and extends preliminary studies.

The demonstration that most of the memory cells in suppressed mice which give rise to Ig-1b DNP-PFC are Ig-1b-bearing cells suggests that the Ig-1b-bearing cells found in spleens of these animals are functional B cells, likely carrying memory for environmental antigens. If this is so, then the demonstration that suppressed and nonsuppressed mice have the same number of Ig-1b-bearing cells may be taken as evidence that immunization with DNP-KLH does not represent a special case in Ig-1b memory development; in other words, that the first stage of memory development for all T-dependent antigens occurs normally in Ig-1b-suppressed mice, despite the absence of the Th required for Ig-1b memory expression.

Avidity of Memory Cells in DNP-KLH-Primed Suppressed and Normal Mice. Julius and Herzenberg (5) have shown by FACS separation of high and low avidity DNP-binding cells that the avidity of the DNP-PFC in adoptive transfer is correlated with the avidity of the receptors on the antigen-binding cells. Therefore, we used the avidity distribution of the DNP-PFC obtained after adoptive transfer of B cells (anti-Thy-1-treated spleen) from DNP-KLH-primed donors as an index of avidity distribution of the memory cells in suppressed and nonsuppressed mice. The transferred B cells (T-depleted spleen cells) were supplemented with carrier-primed T cells from nonsuppressed donors to provide a constant source of Th. Avidity of the DNP-PFC was measured by inhibition by graded amounts of ϵ-DNP-lysine included in the plaquing chamber.

The upper portion of Fig. 1 shows that the distribution of Ig-1b DNP-PFC obtained with memory B cells from suppressed mice is the same as the distribution of Ig-1a and total IgG DNP-PFC and also the same as the avidity distribution obtained with memory cells from nonsuppressed mice (see Fig. 1). The data in the figure are presented as the percentage of DNP-PFC response of each class which is inhibited between the indicated concentration ranges of ϵ-DNP-lysine. Lines 1 and 2 in the figure show that for Ig-1a, Ig-1b, and total IgG (60% IgG_1) obtained from transfer of B cells from suppressed or nonsuppressed DNP-KLH-primed donors, roughly half of the DNP-PFC are inhibited between 10^{-6} and 10^{-7} ϵ-DNP-lysine; the remainder are distributed over the entire range of inhibition concentrations, and show no significant differences between immuno-

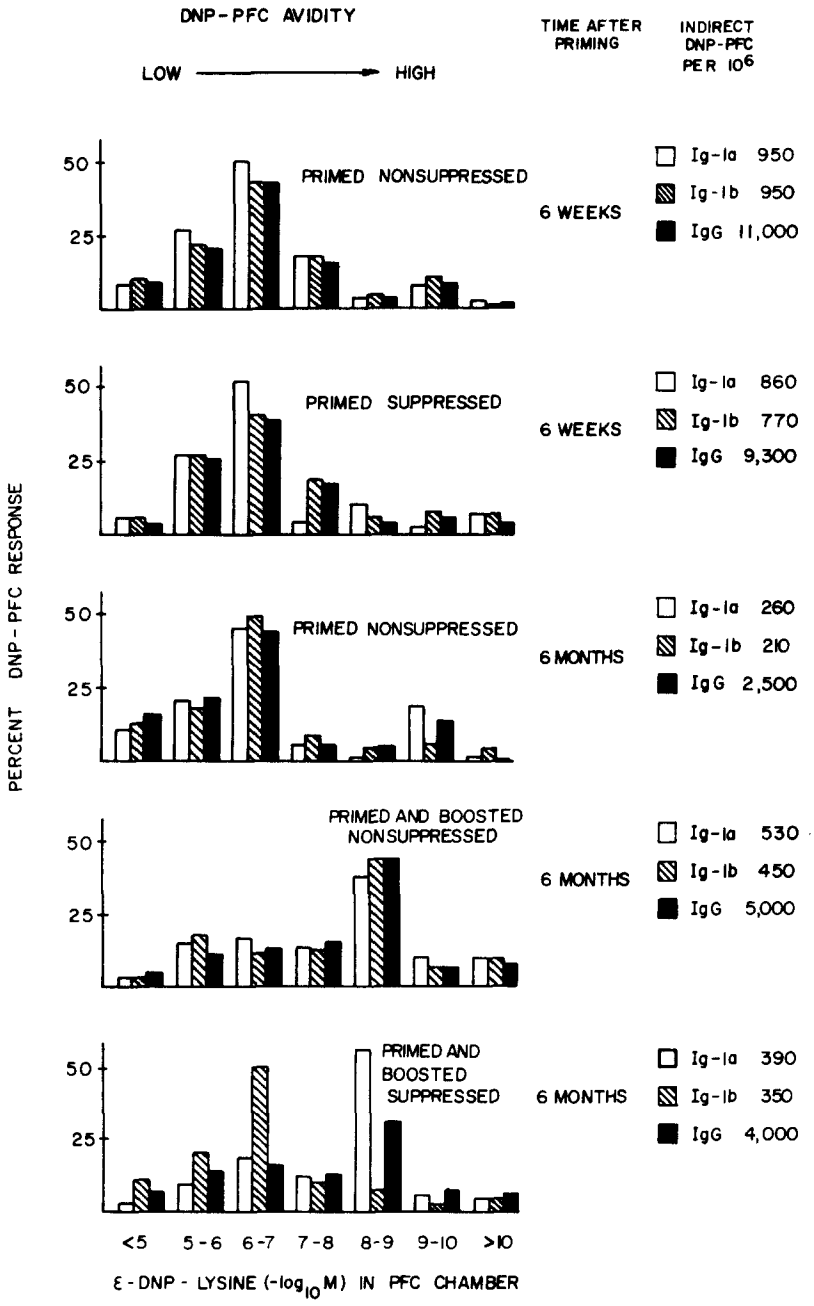

FIG. 1. Selective failure of avidity maturation of Ig-1b memory B cells in allotype-suppressed (Ig-1b Th depleted) mice. Spleen cells from hapten-primed or primed and boosted donors were treated with anti-Thy-1 to remove T cells, combined with spleen cells from carrier-primed donors, and transferred to irradiated recipients. The recipients were boosted with hapten-carrier conjugate (DNP-KLH) on the day of transfer and tested 7 days later for splenic DNP-PFC using the Cunningham chamber PFC assay. ε-DNP-lysine was incorporated into the suspending medium at the indicated concentration before placing cells in the PFC chamber. Results of inhibition assay are expressed as percentage of PFC obtained without inhibitor present. Actual responses (PFC per 10^6 recipient spleen) in the absence of inhibitor are presented in the right-hand panel.

globulin classes or between suppressed and nonsuppressed mice. Thus in DNP-KLH-primed mice, whether suppressed or nonsuppressed there are no detectable differences between Ig-1b memory cells and other memory cells.

Lines 3 and 4 in Fig. 1 show that the memory avidity distribution remains stable for many months after priming. The memory cells used to obtain the DNP-PFC avidity distributions shown in these lines were from nonsuppressed donors primed 6 mo previously and were approximately 8 mo of age at testing.

Avidity of Memory Cells in Primed and Boosted Suppressed Mice. The overall similarity in avidity distribution of Ig-1b and other IgG memory cells found in suppressed and nonsuppressed mice after a single priming injection of DNP-KLH breaks down when memory cell donors are boosted with DNP-KLH several times before testing. Ig-1b memory cells in suppressed mice fail to mature with respect to avidity in comparison with other IgG memory cells.

Lines 4 and 5 in Fig. 1 show that boosting nonsuppressed mice causes a 100-fold increase in the avidity of the Ig-1a, Ig-1b, and IgG memory cells and that boosting suppressed mice causes a similar increase in Ig-1a and IgG memory cells. But despite the increase in avidity of the bulk of the IgG memory cells, and despite the presence of high avidity antibody (produced by antibody-producing cells arising from these memory cells during the course of immunization), the pool of Ig-1b memory cells in suppressed donors does not shift towards a larger proportion of high avidity cells. The avidity of the Ig-1b memory cells in the suppressed mice remains the same as in primed but unboosted donors. Thus the absence of Th capable of helping Ig-1b memory cells also prevents their maturation with respect to avidity.

Discussion

The evidence from studies presented in this publication and from earlier studies by others on B-cell memory in T-deficient mice (1–3) suggests that the development of memory B cells in normal mice occurs in two sequential stages. These differ both in the nature of the changes in the memory cell population and the requirement for interaction with mature Th. The first stage is apparently completed within several weeks after priming and results in a large increase in the number of memory B cells capable of differentiating to cells producing IgG antibody reactive with the priming antigen. The second stage requires more time and additional "booster" antigenic exposure. It is characterized by a shift within the memory cell population so that the relative frequency of high avidity memory cells is substantially increased while the absolute number of memory cells remains essentially the same.

The first stage of memory development appears to be T independent. Memory B cells are induced by antigenic priming in nu/nu and in ATX-BM mice (1–3); in addition, as we have shown here, Ig-1b memory cells are similarly induced in allotype-suppressed mice which lack the specific Th required for Ig-1b memory cell expression [see introduction and accompanying publication (6)]. Thus these data taken together suggest that the mature Th which help memory cell differentiation and expansion to AFC do not appear to play any role in the appearance of the IgG memory cells whose expression they help.

This conclusion is supported by evidence from the avidity studies reported

here which show that Ig-1b Th depletion does not alter the avidity distribution of the Ig-1b memory B-cell pool in primed mice. Ig-1b memory cells from nonsuppressed or suppressed (Ig-1b Th depleted) donors primed with a single dose of DNP-KLH give rise to DNP-PFC with the same distribution of avidities, as measured by ε-DNP-lysine inhibition of PFC formation. Furthermore, the Ig-1b avidity distribution is the same as the Ig-1a and IgG_1 avidity distribution obtained with memory cells from either type of donor. Thus the development of the pool of Ig-1b memory cells in suppressed mice (first-stage memory) appears to be governed by the same mechanism as the development of other IgG memory cells and is unaffected by the absence of Th.

Quite different results, however, are obtained when Ig-1b memory cells are examined in suppressed mice after several booster injections with antigen. Boosting these mice leads to the characteristic shift towards production of higher avidity antibody in Ig-1a and IgG_1 memory. But the avidity of the Ig-1b memory is not changed by boosting. It remains the same as after priming. Since the avidity of Ig-1b memory increases in parallel with Ig-1a and IgG_1 memory in nonsuppressed mice, the selective failure of the Ig-1b memory population to "mature" with respect to avidity in Ig-1b-suppressed mice must be due to the absence of Ig-1b Th.

This Th requirement for B-cell memory avidity maturation (second-stage memory) is consistent with the data from T-cell restoration studies in ATX-BM mice which show that increase in antibody affinity is positively correlated with the availability of T-cell help (4). The argument from these for Th dependence of avidity maturation, however, is considerably strengthened by the data reported here. Suppressed mice, like ATX-BM mice are deficient for Th; but in suppressed mice the deficiency is limited to Th which help Ig-1b memory cells, whereas in ATX-BM mice virtually all helper T cells are missing. IgG memory for all but Ig-1b therefore matures normally in suppressed mice and, unlike ATX-BM mice, suppressed mice have high avidity antibody of all classes except Ig-1b in circulation. This means that "central" factors such as the competition for antigen between serum antibody and cell receptors occurs normally in suppressed mice and can be excluded as the only basis of the T-cell requirement.

Assigning a direct role for Th in avidity maturation does not, of course, exclude antibody competition for receptors as one basic component of the avidity maturation mechanism. The selection and expansion of high avidity memory cell as the result of limited availability of antigen may require both antigen sequestration and T-cell help. In fact, helper T cells might reasonably be expected *a priori* to play a role in such B-cell selection, and this could well be the basis of the Th requirement.

One word of caution in considering the above: the data on Th removal (6) do not necessarily demonstrate that the Th which facilitate Ig-1b memory cell differentiation to Ig-1b antibody-producing cells are the same as those responsible for the avidity maturation of the Ig-1b memory cell pool. We have shown that Ig-1b Th are removed by Ts in Ig-1b-suppressed mice. The simplest hypothesis, which we have opted for here, is that the failure of Ig-1b avidity maturation in suppressed mice results directly from the absence of Ig-1b Th. Nevertheless, it is possible for example, that Ts in these mice also remove another as yet undefined

cell which specifically promotes avidity maturation of Ig-1b memory cells. Thus a number of questions have yet to be resolved before the factors operating in second-stage memory development are well understood.

The data presented here also offer some suggestive insights concerning the nature of first-stage memory development. As we indicated earlier, primary antigenic exposure leads to the appearance of a pool of memory B cells. We have shown elsewhere that these memory cells carry surface IgG determinants which indicate their class and allotype commitment. These memory cells could arise by an antigen driven differentiational change, (i.e., switch) from cells producing (and bearing) IgM or they could arise independently of antigen, exist in small numbers in unprimed animals and merely be selected and expanded by exposure to antigen (22, 23). The Th independence of this stage of memory development, however, makes this latter hypothesis unlikely. If memory arises by selection of IgG-bearing predifferentiated cells, then the expansion of these cells might be expected to require the same type of T help as avidity maturation, which most likely involves just such a selective process. Our data then suggest, although do not prove conclusively, that the first stage of memory involves antigen-driven, T-independent switching to memory cells committed to IgG production and bearing surface IgG indicating its commitment.

Several factors may influence the avidity distribution of memory cells found after priming. The priming protocol could determine which precursor B cells switch to memory cells, and thus determine the avidity distribution of the first-stage memory pool (as well as the class and allotype distribution). Also, the memory cells in primed mice could reflect a combination of first- and second-stage events, depending on how long the priming antigen remains and whether T help is available. In Gershon's and Paul's experiments (4), partially T-depleted mice were primed but not boosted, yet the affinity of the antibody produced increased with time (perhaps due to long-term antigen release). In our studies, priming without boosting gave an initial avidity distribution which was the same in T-depleted and normal mice. No measurable increase in memory cell avidity was detectable without boosting even after 6 mo, suggesting that under our experimental conditions the avidity distribution of the memory cell pool is determined for the most part in the T-independent first stage.

The description of this first stage of memory development as T independent is based on the standard definition of T independence, i.e., it occurs in nu/nu or ATX-BM mice where the absence of the thymus prevents development of mature functional T cells such as Th. Recently, however, nu/nu mice have been shown to have a population of cells which carry some T-specific surface antigens and may be immature T cells prevented from developing further by the absence of the thymus (reference 24, and footnotes 3 and 4). These cells, or for that matter, other, non-T, cells could be required to help in first-stage memory development. Therefore, the description "T independent" should be read as independent of post-thymic T-cell help and should not be taken to imply that the

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B cell necessarily undergoes its antigen driven switch without the help (and therefore the control) of a helper cell.

If the first stage of memory does require help from another cell, it is likely that this cell recognizes a surface marker on the B-cell precursor of the memory cell. Perhaps, to close on a speculative note, IgD immunoglobulins serve this marker function (references 25–27; and B. Pernis, personal communication).

Summary

We present evidence here for two stages in B-memory cell development, the first of which is T independent and the second T dependent. For these studies, we use a new type of T-deficient mouse (allotype suppressed) which specifically lacks T-helper activity (Th) for a subset of memory B cells responsible for approximately 10% of the overall IgG antibody response. We have shown elsewhere that these mice (SJL × BALB/c hybrids suppressed for Ig-1b) lack Th capable of helping Ig-1b memory cells, although they have normal Th activity for all other IgG memory B cells. This selective Th deficiency allows study of the effects of T depletion on memory development and avidity maturation of one population of B cells under conditions where the bulk of the immune response in the animal is proceeding normally, thus obviating environmental problems due to secondary effects of T depletion.

With this system, we show that after a single priming dose of 2,4-dinitrophenyl-keyhole limpet hemocyanin, the memory B-cell pool in suppressed and nonsuppressed donors is indistinguishable with respect to magnitude and avidity of the response for all IgG antibodies produced, including Ig-1b antibody, despite the fact that expression of Ig-1b memory cells is prevented in intact Ig-1b-suppressed mice by the absence of Th capable of cooperating with these memory cells. We have shown elsewhere that virtually all of the Ig-1b memory is carried by Ig-1b-bearing cells.

In contrast with the lack of suppressor T-cell effect on initial Ig-1b memory cell development, our data show that continued Ig-1b memory development is selectively impaired in suppressed mice. When primed mice are boosted repeatedly with the priming antigen, the average avidity of most of the IgG memory cells increases over 100-fold while there is no avidity increase in the Ig-1b component.

To explain these data, we suggest that the development of high avidity memory occurs in two stages. The first stage, which occurs as a result of primary antigenic exposure, is the creation of a pool of IgG-bearing memory cells with a relatively low average avidity for the antigen. The appearance of these first stage memory cells does not require help from (post-thymic) Th, although Th are required for the expression of these memory cells (antibody production). The second stage of B-memory development requires both further antigenic stimulation and B-memory cell interaction with competent Th. This is a continuing process in which the number of memory cells in the pool remains relatively constant but the average avidity of these cells increases with continued antigenic exposure.

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