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Memory B cells at successive stages of differentiation: expression of surface IgD and capacity for self renewal*

In recent studies, we have characterized two memory B cell populations capable of giving rise to IgG antibody-producing cells in adoptive recipients. One population carries surface IgD and gives rise to predominantly low-affinity antibody responses; the other lacks detectable surface IgD and gives rise to predominantly high-affinity responses. These memory populations often coexist in individual donors for long periods of time; however, in strongly stimulated donors, the IgD^+ population is lost after several weeks, and nearly all detectable B cell memory is IgD^- thereafter.
In this publication, we show that the IgD^+ and IgD^- memory populations represent B cells at two successive stages of antigen-dependent differentiation. We used the fluorescence-activated cell sorter (FACS) in a double isolation and transfer protocol to show directly that FACS-isolated IgD^+ memory cells transferred to adoptive recipients give rise both to IgG antibody-producing cells and to an expanded memory population that is predominantly IgD^-. We also show that FACS-isolated IgD^- memory populations from the original donor "self-renew" (*i.e.* give rise to more IgD^- memory) in adoptive recipients and that these events require supplementation of the isolated memory cells with carrier-primed T cells and antigen.

In discussing these findings, we integrate our data with previous evidence on the expression of surface IgG on memory B cells to create an updated view of surface Ig expression during memory development. We also consider these findings in the light of our recent suggestion that the loss of IgD receptors facilitates affinity maturation in the more mature (IgD^-) memory population.

1 Introduction

Memory B cells carry surface IgG that indicate the isotype and allotype commitment of these cells with respect to the antibodies their progeny antibody-forming cells will produce [1, 2]. In addition, some memory B cells still carry surface IgD expressed on the "virgin" B populations from which the memory cells were drawn [3, 4]. The demonstration that IgD^+ memory populations give rise to predominantly low-affinity ($K_a < 10^6$) responses, whereas IgD^- populations from the same donor give rise to substantially higher affinity responses ($K_a > 10^7$), suggested that IgD receptors are lost as memory B cells mature [3, 5]. This hypothesis is confirmed here by double fluorescence-activated cell sorter (FACS) isolation and transfer studies which show directly that IgD^+ memory populations contain the precursors of IgD^- memory cells.

Conflicting views exist in the literature concerning IgD expression on memory B cells. On the one hand, Zan-Bar et al. [6] interpret their FACS isolation and transfer studies as indicating that all memory B cells, defined as cells capable of "self renewal", carry surface IgD. IgD^- cells that transfer IgG responses, they suggest, are end-stage cells programmed for very limited expansion before final differentiation to antibody-forming cells (AFC). Dresser and Parkhouse, in contrast, argue that all memory cells are IgD^- [7]. The IgD that is found on memory populations, in their view, represents residual virgin B cell receptors in the process of being lost.

Our data indicate that neither of these extreme positions is correct. We show that memory B cells, defined by the ability to respond and self-renew in adoptive recipients, occur in both the IgD^+ and the IgD^- spleen cell populations isolated from SJL mice primed 6 weeks prior to separation and transfer. Thus, IgD^- memory cells exist, and IgD receptors can persist on memory cells for relatively long periods and are not necessarily transient.

IgD^+ memory cells, however, are rarely found in strongly stimulated animals [7]. Furthermore, as we show here, they give rise to predominantly IgD^- memory populations when allowed to self-renew in adoptive recipients. Therefore, our studies indicate that the IgD^+ memory population consists of relatively immature cells arrested at a stage in memory development prior to the loss of IgD receptors.

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Abbreviations: **AFC:** Antibody-forming cells **BSA:** Bovine serum albumin **CGG:** Chicken gamma-globulin **CTh:** Carrier-specific helper T cells **DNP:** 2,4-Dinitrophenyl **FACS:** Fluorescence-activated cell sorter **FCS:** Fetal calf serum **KLH:** Keyhole limpet hemocyanin **PBS:** Phosphate-buffered saline **RIA:** Radioimmunoassay **F*:** Fluoresceinated reagent **MEM:** Eagle's minimum essential medium

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2 Materials and methods

2.1 Mice

Two pairs of Ig heavy chain-congenic mouse strains were used: SJL/JHz (Igh^b haplotype) and SJA/9Hz (Igh^a haplotype derived from BALB/c):
BALB/cNHz (Igh^a) and BAB/14Hz (Igh^b from C57BL/Ka).

2.2 Allotype notation

Our previous allotype notation is replaced here by a new notation system recently agreed upon by investigators in the field [8]. Igh-1a and Igh-1b (previously called Ig-1a and Ig-1b) are allotypes of IgG_{2a} isotype Ig; Igh-4a and Igh-4b are IgG_1 allotypes; and Igh-5a and Igh-5b are IgD allotypes. For simplicity, these allotypes are referred to as 1a, 1b, etc., in the text.

2.3 Production of antibody for immunofluorescence and radioimmunoassays (RIA)

Methods used here have all been previously described [3, 9]. Anti-IgG allotype reagents were tested for specific RIA binding to appropriate myeloma proteins. Contaminating antibodies were removed by solid-phase immunoadsorption. ^{125}I-labeled reagents for RIA were labeled by the solid-phase method.

Anti-5b (IgD allotype) was prepared by immunizing SJA with BAB/14 spleen cells. Use of this serum is restricted to SJL mice (because of contaminating antibodies to major histocompatibility complex and other cell surface antigens). Fluorescein-conjugated (F*) anti-5b was prepared using fluorescein isothiocyanate (FITC, Sigma Chemical Co., St. Louis, MO). Anti-IgD allotype antibodies were tested for specificity by binding to Ig on splenic B cells (measured with the FACS). In addition, specificity was determined by immunoprecipitation and gel analysis of radiolabeled spleen cell lysates.

Rabbit anti-mouse brain-associated T cells (BAT) used with guinea pig complement to deplete T cells from splenic populations were prepared as described [10] and absorbed with cells from a B cell tumor [11] until it had no detectable cytotoxic activity against splenic B cells.

2.4 Measurement of allotype representation in antibody responses

Allotype representations in anti-2,4-dinitrophenyl (DNP) hapten responses were measured in an indirect (two-step) solid-phase RIA with DNP coupled to bovine serum albumin ($DNP_{12}BSA$) at 1 mg/ml as the coat antigen and specific ^{125}I-labeled anti-allotype reagents as "second-step" antibodies [9, 12]. A titration of a standard $(SJA \times SJL)F_1$ adoptive secondary antiserum was included in each assay. Results are expressed as units of allotype-carrying antibody relative to the standard (1 unit equals 1% of standard response, *i.e.* for IgG_{2a}, 1 unit $\simeq$ 1 μg anti-DNP/ml serum).

Average values for adoptive secondary responses in experimental groups (3–4 recipients) are shown. Equal amounts of sera from each of the recipients were combined and tested as a pooled sample. Tests of individual recipient's sera show that this method yields an arithmetic average of response in the group. Individuals tend to vary up to about 25% of the pooled value in high responses (~100 units) and as much as 50% of the pooled value in low responses (~20 units).

2.5 Cell preparations

B cells were prepared by incubating whole spleen cells with rabbit anti-BAT for 30 min at 4 °C. The cells were then pelleted through fetal calf serum (FCS), resuspended in guinea pig serum (complement), incubated at 37 °C for 45 min and washed before use. T cell-enriched populations of splenic lymphocytes were prepared by passing spleen cells through nylon wool columns. Erythrocyte-depleted spleens were obtained by incubating spleen cells for 2 min at 4 °C in hemolytic Gey's (NH_4Cl, 0.7%) balanced salt solution. All cell preparations were in Eagle's minimum essential medium (MEM), usually with 5% FCS.

2.6 Hapten priming

B cell donors (SJL) were primed with 100 μg DNP-chicken gamma-globulin (DNP-CGG) or DNP-keyhole limpet hemocyanin (DNP-KLH) or with 800 μg DNP-BSA. Previous studies showed that donors primed in this manner generally have about equal numbers of IgD^+ and IgD^- memory cells [3].

2.7 Carrier priming

T cell donors were primed with 100 μg CGG or KLH. Except where stated, all antigens were injected i.p. as alum precipitates together with 2×10^9 killed *B. pertussis* organisms (Department of Public Health, Boston, MA). Mice were generally used as donors 6 weeks after priming. CGG and DNP-CGG were prepared as previously described [3].

2.8 Immunofluorescence staining and FACS sorting

SJL splenic lymphocytes, harvested in MEM with 5% FCS, were indirectly stained by incubation with Igh^a anti-5b for 15 min at 4 °C and were pelleted through FCS. The cell pellet was resuspended in F* anti-Igh^a (second-step F* reagent) and incubated for a further 15 min at 4 °C. Cells were then pelleted through FCS and resuspended for FACS separation to a concentration of 10^7 cells/ml in MEM with 20 mM HEPES (Gibco, Grand Island, NY). Separations using this procedure have been previously described [3].

Cells were sorted on the basis of the amount of F* anti-IgD (anti-5b) antibody found. Low-angle light scatter (size) thresholds were set so that only small, live lymphocytes were analyzed or separated [13]. In all SJL separations, about 40% of spleen cells spanning the inflection point of the staining profile were discarded. Sorted populations had no detectable contamination with the opposite population, *i.e.* <2%. This protocol has been described previously [3] with presentation of FACS-staining profiles.

2.9 Double-transfer memory assay

FACS-sorted IgD^+ and IgD^- memory B cells, transferred i.v. to adoptive recipients and stimulated with antigen, were allowed to expand and mature for 5 weeks. Progeny populations were then FACS-sorted and transferred to new recipients to reveal the fate of the parents' IgD^+ and IgD^- populations (in the first recipients).

In the first transfer, T-depleted B cells from DNP-primed SJL mice (unseparated, FACS-isolated $5b^+$ or FACS-isolated $5b^-$) were mixed with CGG-primed SJL T cells and 10 μg DNP-

CGG just before i.v. injection into SJL recipients, X-irradiated (800 rd) 18 h previously. Anti-DNP assays performed on sera collected 7 days after transfer were used as an index of the amount of anti-DNP B cell memory present in the DNP-primed donor B cell population.

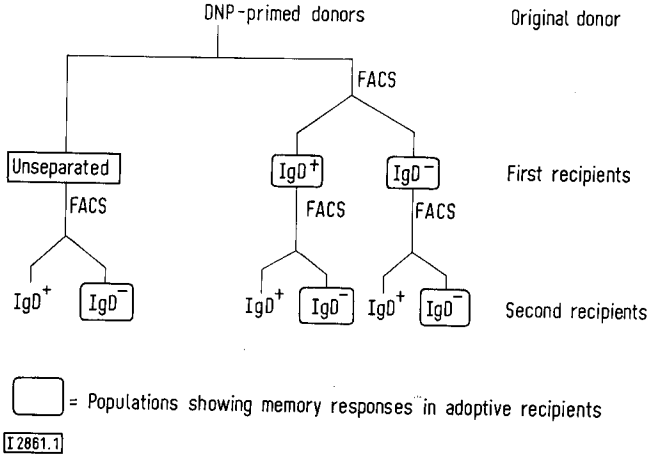
In the second transfer, splenic B cells (unseparated, FACS-isolated $5b^+$ or FACS-isolated $5b^-$), taken from the above adoptive recipients 5 weeks after the first transfer, were mixed with KLH-primed T cells and 10 μg DNP-KLH just before i.v. injection into SJL recipients (X-irradiated, 800 rd, 18 h previously). Anti-DNP assays performed on sera collected 7 days after this adoptive transfer measured the DNP B cell memory derived from the original donor memory populations transferred to the first recipients.

3 Results

3.1 IgD^+ memory cells are precursors of IgD^- memory cells

SJL mice, primed 6 weeks earlier with DNP-KLH, contain two roughly equal-sized populations of DNP-specific memory B cells, one of which is IgD^+ and another IgD^- [3]. These mice were used as B cell donors in the double transfer experiments outlined in Scheme 1. These experiments examine the ability of IgD^+ and IgD^- memory populations to respond in adoptive recipients. In addition, they test the ability of these populations to expand in adoptive recipients to splenic memory B cell populations capable of responding when transferred to a second adoptive recipient. FACS IgD isolations were performed both on memory populations from the original donor and on populations from the various groups of first adoptive recipients to measure transitions between IgD^+ and IgD^- memory cells.

For the first transfer, memory populations (FACS-isolated IgD^+, IgD^- or unseparated B cells) from DNP-KLH-primed donors were supplemented with CGG-primed T cells and DNP-CGG and transferred to irradiated recipients. Control groups received unseparated B cells with unprimed T cells and antigen; T cells with antigen (no B); or B cells and T cells without antigen.



Scheme 1. Double-transfer protocol for determining the fate of IgD^+ and IgD^- memory cells. All populations were T-depleted prior to transfer. Carrier-primed T cells and antigens were supplied to all recipients (except in control groups not shown on this diagram).

Solid-phase RIA analysis of anti-DNP activity in sera collected 7 days after the first transfer are shown in Table 1. The RIA conditions used here, in contrast to those we have used to study affinity maturation [5], are not informative with respect to the average affinity of the anti-DNP responses measured. Comparative studies with both assays, however, indicate that the assay conditions used here detect responses with average affinities (K_a values) at least as low as 5×10^5 and are thus suitable for measured adoptive responses from IgD^+ cells (since such responses have average K_a values that cluster around 10^6 [5]).

As data in the table show, only those control groups that received B cells, carrier-primed T cells and antigen gave rise to an anti-DNP response. Groups that received IgD^+ and IgD^- B cells generated similar responses indicating that both the IgD^+ and IgD^- populations contained memory cells capable of transferring anti-DNP responses and that these populations in the original primed donor were roughly equal in size (assuming equal expansion capability and equal efficiency of detection of antibodies produced in the response).

Table 1. IgD^+ and IgD^- memory populations transfer IgG responses to adoptive recipients

DNP 1° B cells[a]	Nylon T cells[b]	Antigen[c]	IgG anti-DNP in first recipients[d]
FACS-IgD^+	Carrier 1°	+	60
FACS-IgD^-	Carrier 1°	+	65
Unseparated	Carrier 1°	+	80
Unseparated	Carrier 1°	−	<1
Unseparated	Unprimed	+	<1
—	Carrier 1°	+	<1

a) DNP-KLH-primed (1°) SJL T-depleted spleen cells (yield from 10^7 spleen/recipient).
b) CGG 1° nylon-passed spleen (5×10^6/recipient).
c) Ten μg of DNP-CGG used per animal.
d) Igh-1b and Igh-4b responses were approximately equal. Data for Igh-1b responses are shown.

In a series of experiments 5 weeks after the first transfer, FACS-separated IgD^+, FACS-separated IgD^- and unseparated B cells were obtained from each responding group and transferred together with KLH-primed T cells and DNP-KLH to "second recipients". Similar transfers were made from non-responding "first recipient" groups, except that FACS separations were omitted. Responses obtained, measured 7 days after transfer, are shown in Table 2.

Data presented in Table 2 (representative of two double-transfer experiments) show that both IgD^+ and IgD^- memory populations in the primed donor gave rise to predominantly IgD^- memory populations in the first recipients. Virtually no IgD^+ memory responses were obtained from these first recipient groups, except for those ascribable either to the primary responses derived from the supplementary carrier-primed T cells in second-transfer recipients or to responses from memory B cells generated from the initial supplementary T cells in the first recipients. (These possibilities cannot be distinguished since T cells supplemented with splenic B cell populations

from first recipients of carrier-primed T cells plus antigen (no B) give rise to responses in the same range as the supplementary carrier-primed T cells transferred alone into the second recipients; see Table 2). Thus, we conclude that IgD^+ memory cells give rise to IgD^- memory cells, and IgD^- memory cells give rise to more IgD^- memory cells.

Table 2. IgD^+ memory cells become IgD^- after 5 weeks in first-transfer recipients

First isolation and transfer[a)]	Second isolation and transfer[a)]	IgG anti-DNP[b)] in 2nd transfer recipients
FACS IgD^+	FACS IgD^+	12
	FACS IgD^-	75
	Unseparated	61
FACS IgD^-	FACS IgD^+	5
	FACS IgD^-	63
	Unseparated	71
Unseparated	FACS IgD^+	19
	FACS IgD^-	68
	Unseparated	70
No B (T only)	Unseparated	12

a) DNP-KLH-primed B cells, T-depleted and supplemented with CGG-primed nylon T and DNP-CGG for first transfer and KLH-primed nylon T and DNP-KLH for second transfer. In all cases, irradiated animals received the yield from 10^7 DNP-primed spleen or first-recipient spleen and 5×10^6 carrier-primed T and were challenged with 10 μg aqueous DNP-KLH or DNP-CGG at time of transfer. The IgG response for T cell controls (no B cells) in second recipients was between 5 and 10 units.

b) Relative Igh-1b and Igh-4b responses, in units, were approximately the same in all groups. Data shown is for Igh-1b; 1 unit ≃ 1 μg antibody/ml recipient serum. Igh-4b units ≃ 4 μg antibody/ml.

The extent to which the original donor memory populations expand in the first recipients is difficult to estimate, but the yields of memory cells from first-transfer recipient spleens indicate a minimum 20-fold expansion of both IgD^+ and IgD^- in all recipients supplemented with T cells and antigen. This estimate is arrived at as follows: in both the first and the second transfer, FACS-isolated memory cells from roughly 1/20 of a spleen were injected into recipients. Since responses in first recipients and second recipients were roughly equal, the memory cells from the original donor must have expanded at least 20-fold in the first recipients. This estimate is minimal (since not all of the progeny of the memory cells transferred should be recoverable from recipient spleens), and, being based on comparative recovery of memory activity, is independent of the actual number of memory cells transferred, the rate of memory cell expansion, or the amount of antibody produced per memory cell. We therefore conclude that memory populations derived from both IgD^+ and IgD^- populations undergo major expansion; however, because predominantly IgD^- memory cells are recovered from the IgD^+ memory cells, we cannot state whether IgD^+ cells expand as such or first differentiate to IgD^- and then expand.

3.2 Memory B cell expansion requires antigen and help from carrier-specific helper T cell populations

In the double-transfer experiments described above, unseparated (IgD^- + IgD^+) memory cells transferred in the absence of antigen and carrier-primed T helper cells (CTh) generated neither antibody responses (see Table 1) nor detectable memory populations in first recipients (see Table 2). A conservative interpretation of these data allows the conclusion that CTh and antigen were clearly required for expansion of the IgD^- memory populations, *i.e.* if the IgD^- memory cells had expanded, memory would have been detected in the second transfer. The absence of response in the first recipient, however, does not necessarily indicate that IgD^- differentiation to AFC requires CTh since response also requires memory cell expansion. The absence of CTh to support this expansion is therefore sufficient to explain the response failure.

CTh requirements for IgD^+ cells are more difficult to resolve from the double-transfer data because predominantly IgD^- memory populations are recovered from IgD^+ precursors. The failure of the unseparated population to respond indicates that IgD^+, like IgD^- cells, do not generate substantial memory in the absence of CTh and antigen; but since IgD^+ differentiate to IgD^- and IgD^- require CTh and antigen for expansion, the data do not permit resolution of whether IgD^+ directly require CTh help or whether this requirement is indirect, stemming from a requirement for subsequent expansion of IgD^- cells that arise, independently of CTh help, from a nonexpanded population of IgD^+ cells. The IgD^+ to IgD^- differentiation, however, is most likely T-dependent since it appears at least to require help from a population of T helper cells depleted from allotype-suppressed mice [5].

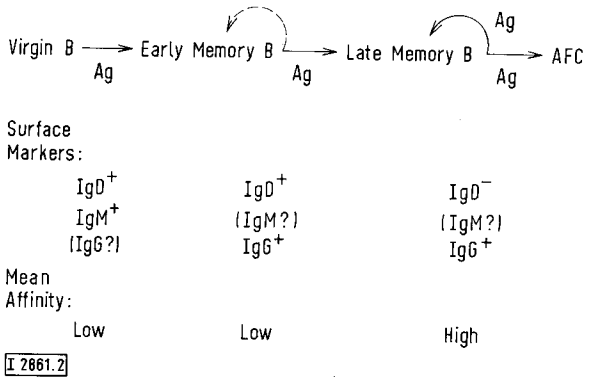
In summary, we conclude from the double-transfer experiments described above that IgD^+ memory cells give rise to IgD^- memory cells; that IgD^- memory cells give rise to more IgD^- memory cells (*i.e.* are self-renewing), but do not give rise to detectable IgD^+ populations; and that at least some of these events are antigen-driven and require help from CTh.

4 Discussion

The differentiation of B cells, from stem cells in the bone marrow to IgG AFC in peripheral organs, proceeds through a series of stages identifiable by changes in the surface Ig expressed by the progressively more mature cells [14]. The early stages in this pathway, up to the appearance of the so-called virgin B cell, occur independently of antigenic exposure. Later stages are antigen-driven and, as we have shown here, are regulated by the availability of help from T cells. Other B cell lineages that may have different differentiation sequences have been identified [15]; however, our discussion here will be restricted to consideration of the differentiation of B cells that participate in T-dependent IgG responses. (We use the term "B cells" here in this restricted sense.)

The diagram in Scheme 2 shows the surface Ig isotypes that mark B cells at different stages in the antigen-dependent B cell differentiation sequences. Virgin B cells, which carry surface IgD, appear to arise directly from the IgM-bearing immature B cells [14]. These virgin cells apparently express the IgM surface marker characteristic of their precursors. If virgin B

cells also express IgG, the amount expressed either must be below detectability, or the entire population capable of giving rise to IgG memory cells must constitute a tiny fraction of the splenic $IgM^+ IgD^+$ population, since very few cells with detectable surface IgG are present in adult spleen, and many of these must be IgG memory cells [1, 2].



Scheme 2. B cell differentiation pathway for IgG AFC: expression of surface Ig.

Two antigen-dependent stages of B cell differentiation have been described here (see Scheme 2). These occur between the virgin B cell and the IgG AFC. We characterize cells at both of these stages as memory B cells because they appear as expanded populations after antigenic stimulation and rapidly give rise to heightened IgG responses on restimulation. The first of these memory cell populations carries surface IgD. It can persist indefinitely in weakly stimulated animals; however, when strongly stimulated, it generally gives rise to mature self-renewing IgD^- memory cells that can be selectively expanded to increase the average affinity of antibody produced by the responding memory pool [5].

IgD^- and IgD^+ memory cells have both been shown to carry surface IgG which indicates the heavy chain commitment of the memory cell and its progeny AFC. The demonstration of IgG on IgD^- cells, and the commitment of these cells to the surface IgG isotype they express, derives from FACS isolation and adoptive transfer studies with memory B cells from strongly stimulated, long-term-primed donors [1, 2]. Studies reported elsewhere [5] show that such donors carry mainly IgD^- memory. The demonstration of IgG on IgD^+ memory cells derives from FACS isolation studies with allotype-suppressed donors which showed that all memory cells capable of giving rise to Igh-1b responses carry surface Igh-1b [1]. Donors used in these studies were similar to donors recently shown to have at least half their memory cells blocked at the IgD^+ stage of differentiation [5].

Our conclusions here are supported by data from other studies using an entirely different approach to the identification of surface isotypes on memory B cells. Coffman et al. [16] have shown that stripping of surface IgG with anti-IgG antibodies prevents a substantial portion of memory cells from binding a radiolabeled antigen that kills the cells to which it binds. Thus, many of the memory cells studied must have exclusively carried IgG antigen receptors. The rest of the memory cells were protected by stripping both IgG and IgD, but not by stripping IgD only. These memory cells must have had both IgD and IgG receptors. Donors for these studies were primed with a protocol which would be expected to yield both IgD^+ and IgD^- memory cells [5, 16].

The IgD^+ and IgG^+ memory cells also probably carry IgM on their surface since virtually no detectable $IgD^+ IgM^-$ cells are found in spleen. Preliminary data from FACS isolation studies with monoclonal anti-IgM antibody suggest this is the case and that IgD^- memory cells have also lost IgM (T. Tokuhisa, J. Haaijman, L. A. Herzenberg; manuscript in preparation). We have therefore tentatively indicated this distribution for IgM receptors on the memory populations shown in Scheme 2.

Data represented here, showing that both IgD^+ and IgD^- memory cells self-renew (expand) in adoptive recipients to generate memory populations that transfer memory to new adoptive recipients, conflict with double-transfer studies reported by another laboratory [6] which have been interpreted as indicating that only IgD^+ memory cells have the capacity for self-renewal in adoptive recipients. We believe that certain surprisingly important technical details are responsible for the failure to detect the self-renewal capacity of IgD^- memory cells in these latter studies:

Zan-Bar et al. [6] used a hapten-carrier conjugate (DNP-BSA) for priming which tends to yield mainly IgD^+ memory cells [7] that give rise to low-affinity adoptive responses [6, 17]. The predominance of the IgD^+ population, however, appears to have gone undetected when FACS-isolated IgD^+ and IgD^- cells from primed donors were transferred to the first adoptive recipients because the Farr assay used to measure recipient responses tends to substantially underestimate the amount of low-affinity antibody present in a serum [17]. This problem, we believe, resulted in a faulty estimation of the relative numbers of IgD^+ and IgD^- memory cells derived from the initial donor and thus probably to the transfer of many more memory cells from first recipients of IgD^+ cells than from first recipients of IgD^- memory cells. FACS separations were not performed on cells transferred from first to second recipients; therefore, the transition from IgD^+ to IgD^- that most likely occurred in first recipients of IgD^+ cells went unnoticed. These problems, possibly compounded by the very high doses of antigen given to both first and second recipients, appear to have been responsible for the erroneous conclusion that IgD^- memory cells cannot generate transferable populations of memory cells in adoptive recipients.

In our studies, the solid-phase RIA conditions were capable of detecting the low-affinity memory responses ($Ka < 10^6$) obtained from IgD^+ cells 7 days after transfer [5, 12]. Priming protocols were selected to yield donors in which the memory pool was split roughly equally between IgD^+ and IgD^- memory cells. Thus, we were able to obtain a somewhat more representative view of the memory B cell maturation pathway and to establish the precursor-progeny relationships described here.

The disparity between results obtained in our studies and in Zan-Bar's has introduced some confusion into the literature; however, it has also had a salutory effect in that it led us to an in-depth analysis of the factors influencing the generation and maintenance of IgD^+ and IgD^- memory populations and thence to the recognition of a probable role for IgD receptors on memory cells [5].

Briefly summarized, these studies have shown that strong antigenic stimulation results in predominantly IgD^- memory populations while weaker stimulation results in the maintenance of both IgD^+ and IgD^- memory. Adjuvants, carrier proteins, timing, genetic factors and the availability of T cell help all influence the extent to which IgD^+ memory cells persist. Thus, by manipulation of these variables, memory populations with widely varied proportions of the two types of memory populations can be obtained.

Surprisingly, regardless of the priming conditions or the extent of transition from IgD^+ to IgD^- memory, these memory populations show one overriding similarity: the IgD^+ memory cells always give rise to predominantly low-affinity adoptive responses while the IgD^- cells consistently generate responses with substantially higher average affinities. This affinity differential, however, does not appear to be due to the selective differentiation of higher affinity IgD^+ memory cells to IgD^- since the IgD^+ memory population, even when it represents only a small proportion of the memory population in a strongly stimulated animal, is still capable of generating quite high affinity responses after several weeks in an adoptive recipient. These data suggest that IgD^+ memory cells differentiate to IgD^- more or less randomly with respect to affinity and that affinity maturation occurs by selective expansion of the higher affinity IgD^- memory cells.

Carrier-specific helper cells and antigen, as we have shown here, are required for expansion of the IgD^- memory population. Since antigen is also likely to be required for expansion and differentiation of IgD^+ populations, these results suggest that the IgD^+ and IgD^- memory populations have different sensitivity thresholds for antigenic stimulation, *i.e.* that low-affinity IgD^+ memory cells can be triggered to differentiate to IgD^- by antigen concentrations that will not allow these cells to be expanded once they lose their IgD receptors.

This differential sensitivity can be explained by taking into account the greater density of surface Ig receptors on IgD^+ memory cells. Both the IgD^+ and IgD^- memory cells have surface IgG receptors; however, these receptors appear to be present at a much lower density than the IgD receptors. Thus, loss of IgD receptors should render the cell less capable of capturing antigen and hence more sensitive to limiting antigen concentration.

This mechanism allows the maintenance of IgD^+ memory populations at relatively low affinity while IgD^- populations are actively being selected for high-affinity response. Thus, it allows the maintenance of a versatile memory population potentially capable of greater diversity of responses to antigens related to the original stimulus without sacrificing the ability to make high-affinity antibody to the stimulus itself. The flexibility that such a system gives to humoral immune responses, we suggest, could account for the evolution of IgD receptors and the complex regulatory processes involved in memory development and expression (see [5]).

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