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Surface Markers and Functional Relationships of Cells Involved in Murine B-lymphocyte Differentiation*

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Lymphocyte precursors originating in the bone marrow undergo several antigen-independent differentiational steps that result in commitment both to eventual function and to antigen reactivity. Exposure of these committed precursors to antigen induces further differentiation, either directly to effector cells or to memory cells capable of rapid expansion and differentiation to effector cells upon reexposure to antigen. The latter stages in these pathways are regulated by other lymphocytes which act singly or in concert to govern the extent of the overall response. Taken together, the regulatory cells and the cells along the developmental pathway from precursor to effector for a given response constitute what may be termed a *network* (Jerne 1974).

Each of the cells within a given network appears to carry one or more distinguishing surface markers. In some cases, these markers derive directly from the functional commitment of the cell. For example, the surface IgG found on memory B cells that give rise to antibody-forming cells (AFC) indicates the class and antigenic specificity of the antibody that will be produced by the progeny AFC. In other cases, the function of the surface marker itself is obscure, but its presence indicates the functional role of the cell in the network. The use of surface markers for the identification of functional subsets of lymphocytes has provided one of the major tools for charting the cellular interactions that result in immune responses.

In this discussion we will focus on the network of cells involved in production of IgG antibody to the dinitrophenyl (DNP) hapten, starting with the effector (antibody-producing) cell and working backward through the developmental sequence. Although there is still more to be learned about the cells in this network, we can at this point draw a sufficiently detailed outline to allow some useful generalizations, primarily with respect to antibody formation, but also perhaps for immune responses as a whole.

Figure 1 shows the functional relationships and surface markers of the cells involved in the latter stages of development of IgG AFC. The diagram is drawn for IgG AFC in general. Any given AFC produces only a single structural IgG species and hence only a single IgG H-chain class and a single set of variable regions. In an allotype heterozygote, the IgG molecule produced carries the allotype controlled by one of the two parental allotype alleles at the H-chain locus expressed.

The immediate precursors of the AFC are IgG-bearing memory B cells. Expansion and differentiation of these memory cells to AFC require exposure to antigen (e.g., DNP coupled to a suitable carrier) and help from T cells (T_H) obtained from carrier-primed donors. T_H are positioned to play a pivotal regulatory role in this process since the expression of B-cell memory (i.e., the differentiation and expansion to AFC) does not proceed measurably without help from T cells. Through the agency of these cells, the potential for antibody production may be increased or decreased without direct interaction with the memory B-cell population. Of course, expression of this potential will be manifested only if memory B cells are present.

Suppressor T cells, at least in some cases, utilize the regulatory pressure point provided by T_H to prevent or decrease IgG antibody production. Allotype suppressor T cells have been shown directly to produce soluble suppressive factors (T_SF) capable of removing T_H activity from carrier-primed spleen cells (Herzenberg et al. 1976). Carrier-specific T_SF also appear to attack T_H (Tada and Taniguchi 1976). We have drawn the T_H removal mechanism of suppression in Figure 1; however, we do not wish to imply that this is the only way in which T cells suppress antibody formation. The different cells in this network are discussed in the sections to follow.

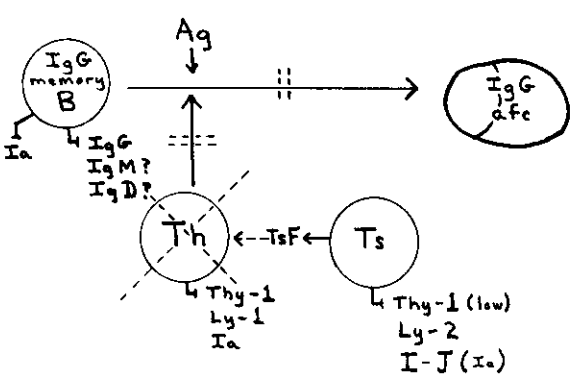

Figure 1. Expression of memory B cells.

* Including the description of IgM and IgD allotypes.

Memory B Cells

The data in Table 1 show that IgG memory cells carry surface IgG that indicates the class and allotype commitment of the memory cell. Using the fluorescence-activated cell sorter (FACS), we have isolated Ig-1b allotype-bearing lymphocytes from DNP-primed donor spleen cell suspensions. (The Ig-1b allotype is found on IgG2a immunoglobulins.) The hapten-primed donors used for these studies were Ig-1a/Ig-1b heterozygotes; therefore, the unseparated population transferred into adoptive secondary recipients gave rise to both Ig-1a and Ig-1b AFC (as well as IgG1 AFC).

The isolated Ig-1b-bearing cell population gave rise only to AFC producing antibody carrying the Ig-1b allotype. These cells did not give rise to Ig-1a or IgG1 AFC. The residual population (Ig-1b-depleted) gave rise to AFC producing all the other IgG antibodies produced by the unseparated spleen cell preparation, including Ig-1a, but did not give rise to Ig-1b AFC (see Table 1).

Similar results were obtained by isolating total IgG2a- or IgG1-bearing cells from DNP-primed donors (Okumura et al. 1976b), i.e., the isolated population gave rise to AFC producing the same type of IgG antibody used to isolate the cells. Thus the precursors of IgG AFC are IgG-bearing memory cells, and the IgG on the surface of the memory cell indicates the class and allotype of the progeny AFC.

As a corollary, this evidence demonstrates that memory B cells, like their progeny AFC, are committed with respect to class and allotype, at least within the confines of the adoptive transfer assay used here. Thus allelic exclusion (the process whereby only one of the two parental alleles is expressed in the immunoglobulin produced by a given AFC) must occur during the differentiation of the memory cell from its immediate precursor or at a stage prior to this differentiation.

Memory B cells carry a number of other surface markers, including Fc receptors (Parish 1975), Ly-4 (McKenzie 1975), and Ia determinants (McDevitt et al. 1976). We have indicated the presence of the Ia determinants in Figure 1 because they may play a role in the communications between functionally different cells within a network (see below).

Helper T Cells (T_H)

Very little is known about how T_H facilitate differentiation and expansion of memory B cells, except that carrier recognition plays an important role. Extending the findings with soluble helper factors (T_HF) that facilitate IgM responses (Feldmann 1972) suggests that IgG help could be mediated by T_HF carrying determinants controlled by loci in the *I* region of the *H-2* complex, closely linked to (or identical with) the loci that control the B-cell Ia determinants. We have recently presented evidence indicating the presence of *I*-region-controlled surface determinants on T cells required to help IgG responses (most likely T_H) (Okumura et al. 1976c). Whether these determinants are involved in T_H-B transactions or T_H-T_S transactions (or neither) remains to be determined.

T_H carry Ly-1 but not Ly-2 surface markers (Cantor and Boyse 1975a), placing them in the same subclass (Ly-1^+2^-) as T cells that amplify cytotoxic responses (Cantor and Boyse 1975b) and initiate delayed hypersensitivity reactions (Huber et al. 1976). Unlike cytotoxic amplifier cells, however, T_H do not have receptors for antigen-antibody complexes, i.e., T_H are FcR^- (Stout et al. 1976).

We have also demonstrated recently that T_H show specificity for the class and allotype commitment of the IgG memory cells that they help (Herzenberg et al. 1976). In the allotypically heterozygous hybrid with which we work, (BALB/c × SJL)F_1, T_H that help Ig-1a or IgG1 memory cells do not help Ig-1b memory cells, at least under our assay conditions. Similarly, Ig-1b T_H do not help Ig-1a memory B cells. This selectivity on the part of T_H is consistent with earlier observations by Ishizaka and colleagues suggesting that IgE memory cells require IgE-specific helpers (Kishimoto and Ishizaka 1973).

Table 1. Presence of DNP Memory in Population of Ig-1b-bearing Cells from Chronically Suppressed BALB/c × SJL Hybrids

DNP-KLH-primed spleen cells transferred (×10^6)			Indirect DNP-PFC[a]			
description[b]	total number of cells	number of Ig-1b-bearing cells[c]	total IgG	Ig-1b	Ig-1a	IgG1
T-depleted cells stained for Ig-1b	5	~0.04	2970	335	356	980
Dullest 85%[d]	5	<0.0005	2620	31	310	870
Brightest 1%[d]	0.05	0.045	185	198	0	0

Data of Okumura et al. (1976b).

[a] Indirect DNP-PFC/10^6 recipient spleen cells. Direct DNP-PFC (<50) subtracted.

[b] T-depleted cells were prepared by treating spleen cells from DNP-KLH-primed mice with anti-Thy-1 plus complement. Fluorescent Ig-1b cells were obtained by staining the T-depleted cell suspension first with rabbit anti-Ig-1b and then with [F]goat anti-rabbit IgG. After staining, the cells were passed through the FACS to obtain separated fractions. All adoptive transfer recipients of T-depleted spleen cells or separated fractions were given 10^7 KLH-primed spleen cells (helper T cells) from nonsuppressed (BALB/c × SJL)F_1 donors.

[c] Number of Ig-1b-bearing cells as determined by fluorescence microscopy.

[d] Percentage of T-depleted B-cell suspension after anti-Thy-1 treatment.

The demonstration that help for Ig-1b memory-cell expression in (BALB/c × SJL)F_1 hybrids requires a specific subset of T_H committed to help Ig-1b memory cells (Ig-1b T_H) explains how allotype T_S can specifically suppress Ig-1b antibody production by removing T_H activity. T_H appeared to be unlikely candidates for allotype T_S targets, since removal of T_H activity was expected to decrease equally antibody production in all classes and allotypes rather than just Ig-1b. But since Ig-1b T_H activity in this system is distinct from Ig-1a T_H and other IgG T_H activities, it can be selectively removed by allotype T_S.

Suppressor-Helper Interaction

Several types of evidence pointed to Ig-1b T_H as targets for allotype T_S:

1. Normal numbers of functional Ig-1b memory B cells are found in DNP-primed, Ig-1b-suppressed mice (Okumura et al. 1976b; Herzenberg et al. 1974). The specificity of the allotype suppression for Ig-1b at first suggested that T_S were removing Ig-1b B cells, but the demonstration of the Ig-1b memory B cells in suppressed mice argues in favor of a different target for T_S.
2. Ig-1b T_H activity was not detectable in spleen cells from carrier-primed, allotype-suppressed mice. T_S activity, which might have masked the T_H activity, was depleted from the spleen cell suspension before testing for T_H (Herzenberg et al. 1976).
3. Data from titrations of T_S, T_H, and B cells in adoptive secondary transfer assay are best explained by stoichiometric removal of T_H by T_S (Herzenberg et al. 1975b).

The most conclusive demonstration that T_S suppress by removing T_H activity, however, comes from data such as those presented in Table 2. These show that Ig-1b T_H is specifically removed from carrier-primed spleen cells cultured in the presence of a T_SF. This factor is released into the medium ("supernate") by spleen cells from nonprimed, allotype-suppressed donors cultured for 24 to 48 hours. T_H cultured in medium alone or in the presence of control-culture supernates from normal spleen cells show normal Ig-1b T_H activity in an adoptive secondary transfer assay. The Ig-1b and Ig-1a DNP-plaque-forming cells' (PFC) responses obtained with these T_H were equal and constituted roughly 10% of the total IgG response. These responses are similar to those obtained with uncultured, carrier-primed spleen cells. The T_H cultures in the presence of T_SF (suppressed spleen supernate) gave the same Ig-1a and total IgG responses as the control culture; however, these cells gave no measurable Ig-1b response.

The T_SF-treated cells were also tested for Ig-1b suppressive activity to determine whether the treatment had induced T_S activity rather than removed T_H activity. Data in Table 2 show that no T_S activity was demonstrable, although the T_H dose was fixed at a level of maximum sensitivity for the detection of T_S (Herzenberg et al. 1975a). These data also preclude carry-over of T_SF into the adoptive transfer.

In these experiments, T_SF treatment of carrier-primed cells precedes the T_H encounter with memory B cells in the adoptive transfer. Therefore, products from suppressed spleen (T_SF) must remove Ig-1b T_H activity rather than attack B cells. This evidence, taken together with data from other studies showing that serum from suppressed mice contains T_SF or T_SF-like activity (K. Okumura and L. A. Herzen-

Table 2. Absence of Ig-1b Helper T-cell Activity in Ig-1b-suppressed Mice: Suppressor T Cells Removed by Anti-Ly-2.2

	(BALB/c × SJL)F_1 spleen cells transferred (×10^6)						
	DNP-KLH-primed	KLH-primed	KLH-primed suppressed (T_H + T_S enriched)[b]		Indirect DNP-PFC[a]		
Activity tested	B cells[c]	T_H	number treated	treatment[d]	Ig-1b	Ig-1a	total IgG
	6				<10	<10	20
	6	6			290	310	3050
T_H	6	4			200	250	1880
	6		4	NMS	<10	370	3790
	6		4	anti-Ly-2.2	<10	300	3220
T_S	6	2			100	130	900
	6	2	2.5	anti-Ly-2.2	120	300	3000
	6	2	2.5	NMS	<10	370	3690

Data from Herzenberg et al. (1976).

[a] Indirect DNP-PFC/10^6 recipient spleen cells. Direct DNP-PFC (<40) subtracted.

[b] B cells were depleted from spleen cell population by nylon wool passage prior to treatment. T-enriched population had more than 80% T cells and less than 5% B cells (Julius et. al. 1973).

[c] T cells were depleted by treatment with anti-Thy-1 plus complement.

[d] Cells treated with indicated serum plus complement. Number of cells transferred = remainder after treatment of indicated cell number.

berg, unpubl.), suggests that the in vitro system reflects the in vivo mechanism of suppression.

The evidence that T_S remove T_H activity is consistent with evidence reported by Tada and collaborators on the mechanism of carrier-specific suppression (Tada and Taniguchi, this volume). The nature of carrier-specific suppression itself suggests a T_S-T_H interaction, since T_S suppress responses to the carrier protein and to haptens coupled to it. Tada has now shown that splenic T cells, but not B cells, absorb the carrier-specific T_SF which suppress in vitro secondary anti-DNP responses to a DNP carrier conjugate (DNP coupled to keyhole limpet hemocyanin [KLH]) (Tada and Taniguchi 1976). Although Tada has not yet demonstrated directly that the carrier-specific T_SF remove T_H activity, his evidence showing that splenic T cells have a receptor site for T_SF suggests that T_S act on T_H in this system as well.

Suppressor T-cell Surface Markers

Allotype suppressor T cells are generated in (BALB/c × SJL)F_1 hybrids by perinatal exposure of the mice to the Ig-1b (paternal) allotype. Several surface markers have been recognized on these cells. Like other T cells, allotype T_S carry Thy-1 surface antigens; however, the amount of Thy-1 on T_S appears to be considerably lower, both by fluorescent staining and cytotoxicity criteria, than the amount of Thy-1 on most splenic T cells (Herzenberg et al. 1975a; V. L. Sato and L. A. Herzenberg, unpubl.). Allotype T_S also carry Ly-2 surface markers. Since these cells do not carry Ly-1 surface markers, they fall into the $Ly\text{-}1^-2^+$ T-cell subclass, which also contains the precursors of cytotoxic precursor and effector cells. The placing of T_S in the $Ly\text{-}1^-2^+$ T-cell subclass clearly distinguishes them from T_H, which are in the $Ly\text{-}1^+2^-$ subclass.

We have recently shown that T_S carry another surface marker, which is controlled by a locus *(Ia-4)* in a new subregion *(I-J)* of the *I* region of the *H-2* complex. *Ia-4* determinants are selectively expressed on T_S and on some T cells in normal (nonsuppressed) spleen (Okumura et al. 1976a; Murphy et al., this volume). They are not found on T_H or B cells.

Tada has shown that carrier-specific T_S carry determinants controlled in the *I-J* subregion. He has also shown that carrier-specific T_SF carry *I-J*-controlled determinants. We have not yet tested for *I-J*-controlled determinants on allotype T_SF; however, other similarities between the two types of suppression (Murphy et al. 1976; Tada et al. 1976) make it likely that this marker also will be found on allotype T_SF.

Development of High-avidity Memory B Cells

Repeated exposure of mice to booster doses of antigen leads to a significant increase in the affinity of the antibody produced. This increase, at least in part, reflects a change in the composition of the memory B-cell pool towards a greater percentage of memory cells capable of giving rise to AFC producing high-avidity (affinity) antibody in an adoptive transfer assay (see below). Gershon and Paul (1971) have shown that T_H depletion drastically curtails affinity increase. Similarly, as data presented in this section will show, the absence of T_H capable of helping Ig-1b memory B cells specifically prevents avidity maturation of the Ig-1b memory B-cell pool in primed and multiply boosted allotype-suppressed mice. These findings are diagrammed in Figure 2.

Testing the avidity distributions of memory B-cell populations requires transfer of these populations with a single source of T_H in order to avoid possible effects due to differences in T_H-priming. Therefore, T-depleted populations of spleen cells from primed or primed and boosted donors were supplemented with a single pool of carrier (KLH)-primed spleen cells and transferred into adoptive secondary recipients. The avidity distributions of the memory B-cell populations were measured as the percentage of resultant AFC (DNP-PFC) inhibited by graded doses of ε-DNP-lysine (Okumura et al. 1976c).

The first two graphs in Figure 3 show that the same avidity distribution is obtained from 6-week primed donors whether the donors were suppressed or not. The third graph in the figure shows that this avidity distribution is maintained in the absence of further exposure to antigen for at least 6 months. The last two graphs show that three booster injections given subsequent to priming cause roughly a 100-fold increase in the average avidity of the bulk of the memory cells in suppressed and nonsuppressed donors. In the allotype-suppressed donors, however, the pool of Ig-1b memory cells shows no increase in average avidity. Thus the absence of Ig-1b T_H activity in suppressed mice prevents avidity maturation of the Ig-1b memory B-cell pool under conditions where Ig-1a and total IgG antibody are maturing normally in the same mouse.

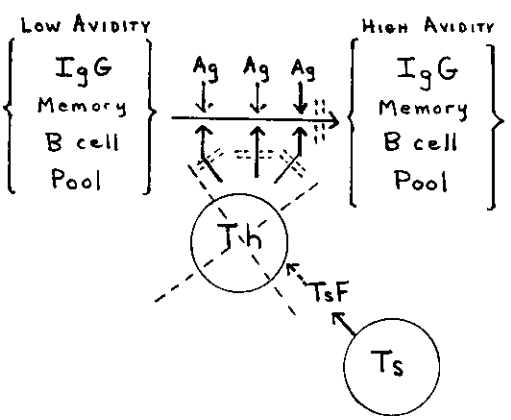

Figure 2. Development of high-avidity memory.

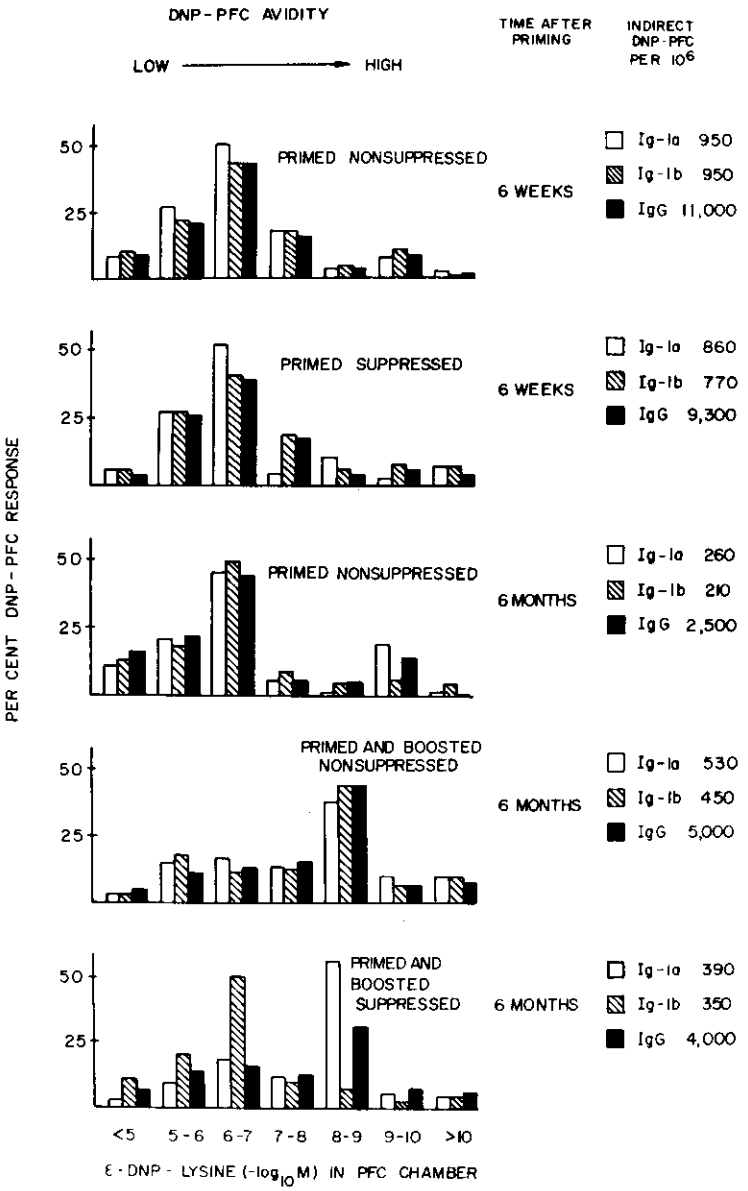

Figure 3. Selective failure of avidity maturation of Ig-1b memory B cells in allotype-suppressed (Ig-1b T_H-depleted) mice. Spleen cells from hapten-primed or primed and boosted donors were treated with anti-Thy-1 to remove T cells, combined with spleen cells from carrier-primed donors, and transferred 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 prior to placing cells in the PFC chamber. Results of inhibition assay are expressed as percentage of PFC obtained without inhibitor present. Actual responses (PFC/10^6 recipient spleen) in the absence of inhibitor are presented to the right. (Reprinted, with permission, from Okumura et al. 1976c.)

Since the absence of Ig-1b T_H does not interfere with the appearance of Ig-1b memory after priming but does prevent the avidity maturation of the Ig-1b memory B-cell pool after boosting, memory B-cell development may be divided into two stages, only the second of which (avidity maturation) requires help from T cells capable of interacting with memory B cells.

Early Stages in Memory B-cell Development

In contrast with avidity maturation, several studies suggest that the appearance of IgG-bearing memory B cells does not require help from mature T cells. The data presented above show that Ig-1b T_H do not participate in the first stages of Ig-1b memory development. Studies with nu/nu mice show that antigenic priming leads to the development of B-cell memory, although that memory cannot be expressed unless the animals are given carrier-primed T cells from a syngenic source (Diamantstein and Blitstein-Willinger 1974; Schrader 1975). Similar results were obtained with bone-marrow-reconstituted, adult-thymectomized, irradiated (ATx BM) mice (Roelants and Askonas 1972). While none of these studies exclude help from primitive (non-thymus-influenced) T cells, they clearly demonstrate that the initial development of IgG memory B-cell populations occurs independently of help from the mature T cells found in normal (as opposed to athymic) animals.

The immediate precursor of the IgG memory cell has not yet been identified. The early lineage of this cell traces to the bone marrow, where early hematopoietic stem cells differentiate through an unknown number of stages to become committed

"pre-B" cells destined eventually to become AFC. At some point (before or after commitment), these cells leave the bone marrow, settle in peripheral lymphoid organs, and finish development to Ig-bearing "B" lymphocytes (see diagram in Fig. 4). Hematopoietic stem cells located in the spleen also give rise to B cells apparently similar to those engendered in the bone marrow.

Ontologic studies show that the first B cells to arise in the developing animal have IgM surface markers. Later, B cells with both IgM and IgD surface immunoglobulins appear. Finally, IgG-bearing cells are found (Owen et al. 1974). In the adult spleen and lymph nodes, all three types of B cell occur, but whether these cells represent stages along the pathway to memory development or independently differentiated B cells has yet to be established.

The existing data suggest that the IgG-bearing cells arise from cells bearing either IgM or IgM and IgD. AFC producing both IgM and IgG, or bearing IgM and producing IgG, have been described (Pernis et al. 1971), and exposure to anti-μ (IgM H-chain) antibody either in vivo (Lawton and Cooper 1974) or in vitro (Kearney et al. 1976) prevents development of IgG-producing cells. These data suggested a precursor relationship between IgM and IgG B cells. The data presented earlier, showing that mature T cells are not required for initial IgG memory development, also argue in favor of a differentiational "switch" from IgM to IgG commitment rather than the selective expansion of IgG precursors differentiating directly from pre-B cells. Since the maturation of avidity (which most likely occurs by selective expansion) requires T-cell help, it seems unlikely that such selective expansion would be T-independent in the initial stages of memory development.

The postulated switch from IgM to IgG commitment most likely in all cases requires antigenic exposure, although it is difficult to rule out antigen-independent switching. In any event, the appearance of measurable numbers of IgG-bearing B cells committed to a given antigen (i.e., memory B cells) requires exposure to antigen under priming conditions.

Surface Markers on Early B Cells

Several new surface markers have been identified recently which may help to clarify the developmental relationships of cells involved in the early stages of B-cell development. One of these, T_HB, appears to be an early differentiational marker which may be present on pre-B cells (Stout et al. 1975). Two others, allotypes of IgM and IgD, mark the early B cells and have already proved useful for demonstrating that the cell-surface immunoglobulins produced by these cells are allelically excluded, i.e., controlled by one or the other of the parental alleles at the IgM or IgD locus (see below and Goding et al. 1976).

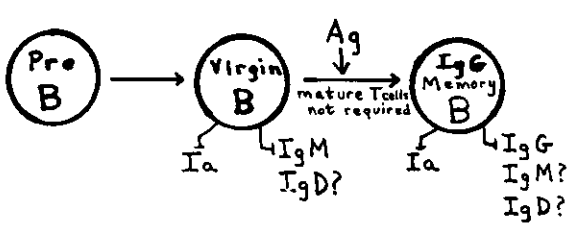

Figure 4. Early stages in B-cell development.

T_HB. The T_HB marker was first identified because of its rather unusual distribution among lymphocyte populations. B cells and about half the cells in the thymus were shown to be T_HB-positive, whereas peripheral T cells were T_HB-negative (Stout et al. 1975). Later studies showed that the T_HB marker disappears as the cells in the thymus mature, i.e., subcapsular cortical thymocytes are T_HB^+, whereas medullary cortisone-resistant thymocytes are all T_HB^-. A parallel maturational sequence appears to take place with B cells; B-cell populations in young (BALB/c) mice have a high surface density of T_HB, whereas B cells in more mature mice have considerably smaller amounts of surface T_HB (Stout et al. 1975; Yutoku et al. 1976).

Two types of mice with genetic defects affecting the immune system (nu/nu and CBA/N) show T_HB abnormalities in that the amount of T_HB on B cells does not decrease with age as it does in BALB/c. A third strain, SJL, also shows this type of T_HB distributional abnormality (R. D. Stout, C. Nottenburg and L. A. Herzenberg, unpubl.).

Recently, a student in our laboratory (L. Eckhardt) demonstrated that a single locus controls the level of expression of T_HB on splenic B cells in adult SJL mice. Her data is presented in Figures 5 and 6. Figure 5 shows the T_HB surface density distribution profiles obtained when spleen cells from BALB/c, SJL, and the F_1 hybrid between these strains are stained with goat anti-T_HB followed by fluoresceinated rabbit anti-goat Ig. (The profiles, obtained by analysis with the FACS, show the fraction of spleen cells stained as a function of the amount of fluorescence on the cell surface.) Experiments in which the fluorescein-stained T_HB populations were counterstained with a rhodamine-reagent surface Ig showed that the T_HB-bearing spleen cells all carry surface Ig, i.e., T_HB-bearing spleen cells are B cells.

Comparison of the SJL and BALB/c profiles shows that while both types of spleen cells have a large negative component (T cells), the SJL T_HB^+ spleen cells stain considerably brighter than the BALB/c. The (BALB/c × SJL)F_1 hybrid spleen-cell profile is intermediate between the two parental profiles and clearly distinguishable from either.

Figure 6 shows the two types of profiles from backcross progeny obtained by crossing the F_1 hybrid to the low (BALB/c) parent. One is indis-

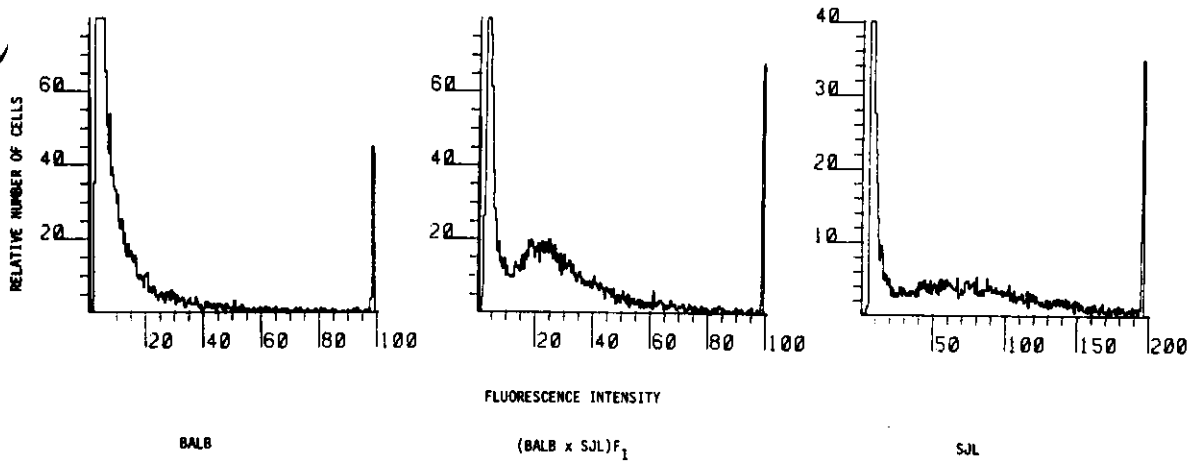

Figure 5. Genetic control of T_HB levels on splenic B cells. FACS profiles showing fluorescence intensity distributions of spleen cells stained with goat anti-T_HB followed by fluorescein-conjugated rabbit anti-goat Ig. In each panel, roughly half the cells show more than 10 units of fluorescence intensity.

tinguishable from the F_1 hybrid profile. So far, 32 backcross mice have been tested. Of these, 17 show F_1 hybrid profiles and 15 show parental profiles. The virtually equal ratio of the two types of profiles in the backcross mice indicates that alleles at a single locus control the level of T_HB expression in these mice. Data from these studies have also shown that the locus controlling T_HB expression is not genetically linked to the *H-2* or Ig chromosomal regions (L. Eckhardt, R. D. Stout and L. A. Herzenberg, unpubl.).

These findings raise a number of questions concerning the nature of the relationship of T_HB control to the immunologic abnormalities associated with SJL mice and the relationship of the T_HB-controlling locus found in the SJL and BALB/c strain combination to the high T_HB expression observed in CBA/N and nu/nu mice. Perhaps the answers to these questions will be more easily obtainable when the functional roles of high-versus-low T_HB-bearing B cells are established and the maturational sequence involving the progressive loss of T_HB from both B cells and thymocytes is clearly defined.

T_HB may also prove useful as a marker for identifying cells in the early stages of the normal lymphocyte differentiation pathway. Since this marker appears in high density both on (immature?) B cells and immature thymocytes, it may possibly be present as well on newly differentiating lymphocytes before their divergence into the T- and B-cell pathways.

IgD and IgM allotypes in the mouse. The discovery of methods for obtaining antibody to allotypes on mouse IgD (Goding et al. 1976) and IgM immunoglobulins (L. A. Herzenberg et al., in prep.) provides powerful new tools for tracing the development of B cells. It is now possible to investigate (1) whether cells bearing IgM only and cells bearing IgM and IgD are of separate lineages, or whether one type of cell is precursor to the other; (2) which type of cell is the precursor of the IgG- or IgA-bearing memory B cell; and (3) whether IgD is found on memory cells. Thus it may soon be possible to obtain a clearer view of the role(s) of IgD immunoglobulins in the immune response.

Unlike other types of immunoglobulins, IgD molecules are found almost exclusively on B-cell surfaces rather than as both surface and secreted immunoglobulins. This suggests a receptor function for IgD, either to turn on, turn off, or modulate (control) B-cell division and/or differentiation.

Heterologous antisera to human IgD immunoglobulins (Rowe et al. 1973) were obtained some time ago. Fluorescent staining with these antisera showed that most human peripheral lymphocytes carry surface IgD as well as IgM (Pernis et al. 1974). Abney et al. (1976) subsequently extended these findings to the mouse, using a heterologous antiserum to mouse IgD. More recently, Goding et al. (1976) discovered anti-IgD allotype antibodies in some C57BL/6 alloantisera made against spleen cells of CBA. We have exploited this general immunization procedure to make a number of antisera that contain antibodies against both IgD and IgM allotypes of mice.

The strategy for producing these sera is to make antibodies in one member of an allotype congenic pair against spleen cells of an unrelated strain with the opposite allotype, e.g., SJA (Ig^a) anti-BAB/14 (Ig^b). Four weekly injections of 10^7 spleen cells intraperitoneally suffice.

These sera also contain antibody to *H-2* and other cell-surface antigens; therefore, to test for antibody reactive with mu (μ) and delta (δ) allotypes without interference from antibody to non-Ig cell-surface constituents, the sera are tested with spleen cells from a strain congenic with the serum producer but carrying the allotype of the immunizing donor. Thus SJL (Ig^b) anti-BALB/c (Ig^a) is tested with SJA (Ig^a) spleen cells. Antibody is detected by fluorescent staining of the target cells in an indirect fluorescent

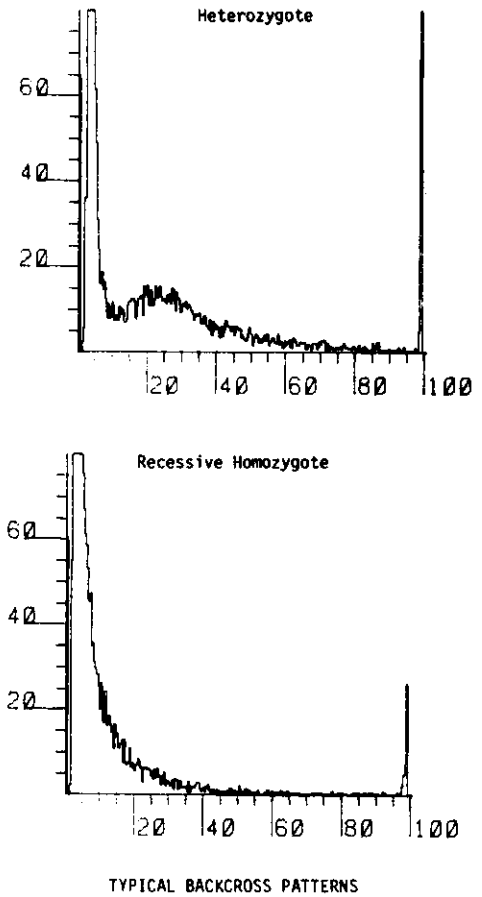

Figure 6. Typical T_HB staining patterns with spleen cells from (BALB/c × SJL)F_1 × SJL backcross progeny. See legend to Fig. 5 for staining details. Of 32 mice, 17 showed the heterozygote pattern and 15 showed the BALB/c (recessive) homozygote.

staining assay using a second step of fluorescein-conjugated anti-mouse IgG. Since only a few percent of spleen cells have surface IgG, the background (second step only) is negligible when compared to the large number of B cells (roughly 40%) that stain with an anti-IgM or anti-IgD first-step reagent. FACS analysis profiles, obtained after staining in this way, are presented in Figures 7 and 8.

The left-hand panel in each of the figures shows the profile for SJL spleen cells stained with an SJA anti-BAB/14 spleen antiserum. Background (second step only) control staining curves are presented in each panel. Profiles for the SJA control superimpose with the "second step only" curves. Comparison with the controls shows that roughly 40% of spleen cells stain with these antisera.

The antisera shown in Figures 7 and 8 were chosen because they represent two types of antisera obtained with this immunization protocol: those containing antibody reactive mainly with IgD (Fig. 7) and those also containing antibody reactive mainly with IgM (Fig. 8). The antibody specificities of these sera are indicated by the profiles in the right-hand panels of the figures, which show the effect of absorbing the antisera with C57BL/10 (Ig^b) serum globulins coupled to Sepharose 4B. This absorbant contains IgM but not IgD.

There is relatively little change in profile obtained after absorption of the serum shown in Figure 7; thus this serum mainly contains antibody against a surface-bound Ig molecule not found in serum (IgD). In contrast, absorption of the serum shown in Figure 8 results in a dramatic shift in the staining profile, indicating the removal of antibody to a serum immunoglobulin also found on the surface of a large number of splenic lymphocytes (IgM). Repeated absorption of this latter serum fails to remove all staining activity, indicating the presence of some antibody to IgD in addition to the antibody IgM. Similar results were obtained with antisera obtained from a reciprocal immunization: SJL (Ig^b) anti-BALB/c (Ig^a) tested on SJA (Ig^a) spleen cells. Thus antisera obtained with this immunization protocol appear to detect allotypes on μ and δ H chains. Confirming evidence is presented below.

The loci controlling the IgD and IgM allotypes have been designated Ig-5 (δ) (Goding et al. 1976) and Ig-6 (μ). The alleles present in BALB/c, following standard type strain notation (Herzenberg et al. 1968), have been designated *Ig-5*^{a} and *Ig-6*^{a}. Those present in C57BL/10 have been designated *Ig-5*^{b} and *Ig-6*^{b}. SJA and C3H.SW carry *Ig-5*^{a} and *Ig-6*^{a}; SJL and C3H.SW-Ig^b carry *Ig-5*^{b} and *Ig-6*^{b}.

The use of congenic strains to define the Ig-5 and Ig-6 alleles was based on the assumption that these loci are part of the Ig H-chain linkage group (Herzenberg 1965) (see Fig. 9). A number of different congenic strains, representing an aggregate of some 50 backcrosses, have now been typed for IgM and IgD allotypes. All show that the Ig-5 and Ig-6 alleles remain in coupling with the Ig-1 through Ig-4 alleles. Thus the presence of the new loci in this linkage is established.

Confirmation that Ig-6a and Ig-6b are IgM (μ) allotypes comes from studies which show that (1) antibody to Ig-6a is absorbed by a BALB/c (Ig^a) IgM myeloma protein, MOPC-104; (2) antibody to Ig-6b is absorbed by a BALB/c-Ig^b (C.B/20) IgM myeloma protein, CBPC-112, kindly provided in advance of publication by Drs. E. Mushinski and M. Potter of the NCI; (3) goat anti-μ antibody cocaps with anti-Ig-6a or anti-Ig-6b; (4) ^{125}I-conjugated IgM myeloma proteins are precipitated by anti-Ig-6; and (5) ^{125}I-surface-labeled proteins precipitated with anti-Ig-6 and run in SDS-urea-PAGE under reducing conditions show a band that co-migrates with μ chain.

The evidence that anti-Ig-5a and anti-Ig-5b detect IgD allotypes, in addition to what has been published (Goding et al. 1976) or presented above, is based on studies which show that (1) rabbit anti-δ antiserum (Abney et al. 1976) blocks all staining

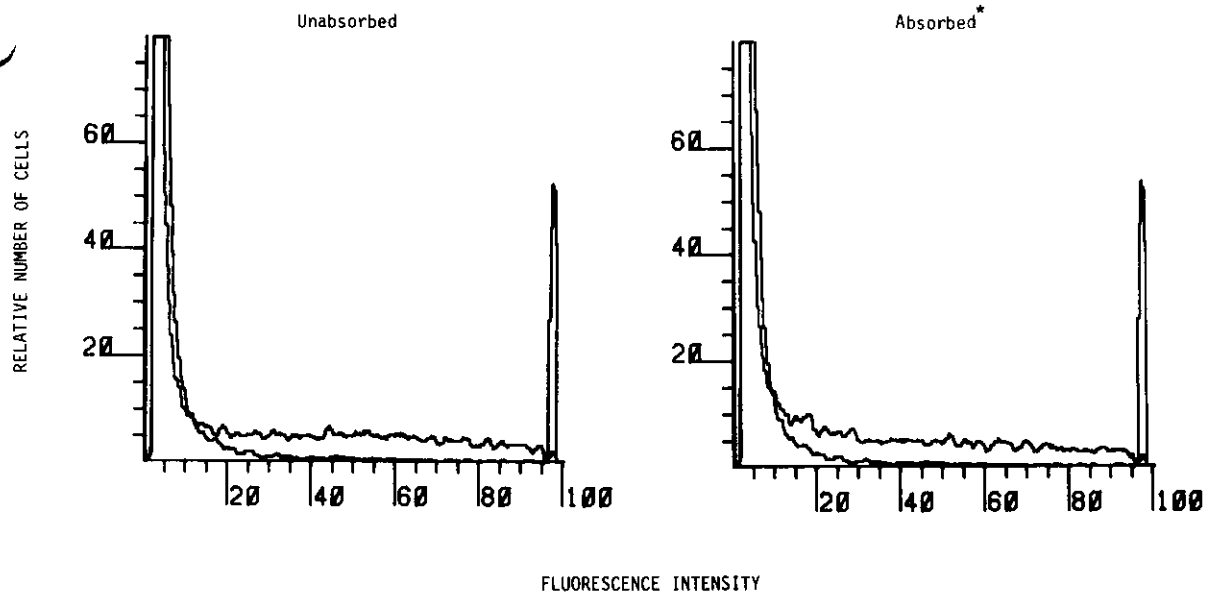

Figure 7. Presence of Ig-5b (IgD allotype) on SJL splenic B cells. Spleen cells were labeled with a selected SJA anti-BAB/14 spleen cell unabsorbed *(left)* or absorbed as indicated *(right)*. Cells were then stained with ^{F}goat anti-mouse IgG (upper curve). Two curves superimposed make up the lower curve: (1) SJA (serum donor) spleen cells stained as above, and (2) SJL spleen cells stained only with ^{F}goat anti-mouse IgG (second step only). Roughly 45% of the spleen cells show greater than 20 units of fluorescence on the upper curve, in contrast to less than 5% on the lower curve. The peak on the far right of each curve represents those cells staining with an intensity greater than 100. The similarity of the upper curves in the left and right panels indicates that absorption (with IgM containing Ig^{b} globulins) does not remove reactive antibody; hence the antiserum is directed against Ig-5b exclusively (see text).

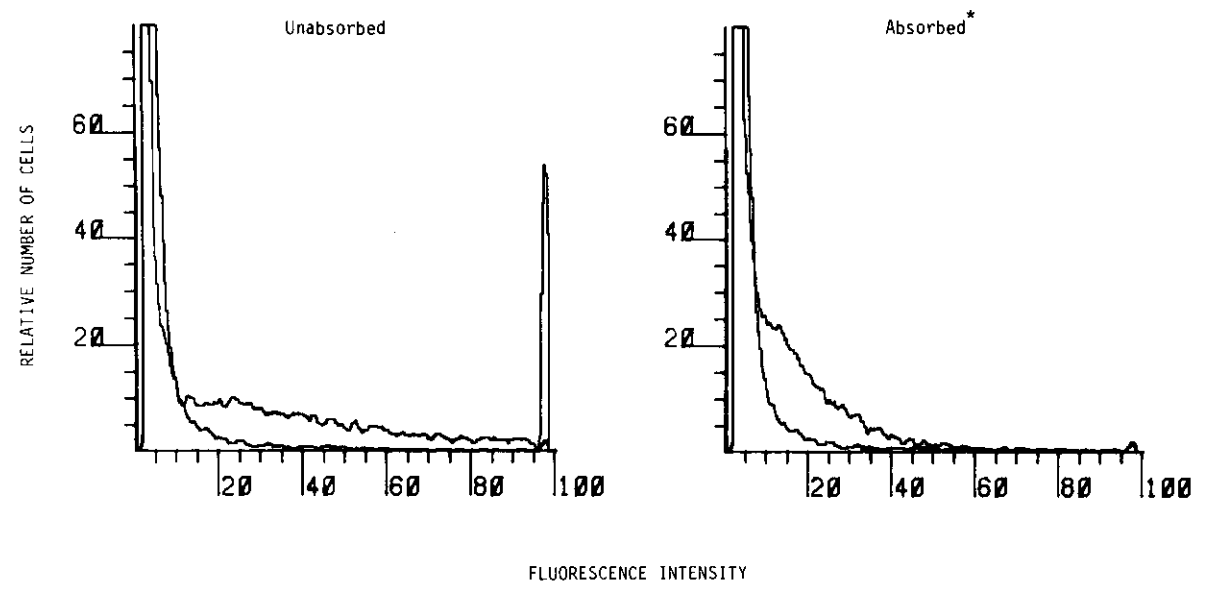

Figure 8. Presence of Ig-6b (IgM allotype) on SJL splenic B cells. SJL spleen cells were stained as described in the legend to Fig. 7; however, this lot of antiserum reacts predominantly with Ig-6b, as shown by the marked decrease in staining intensity obtained after the serum is absorbed with IgM containing Ig^{b} globulins.

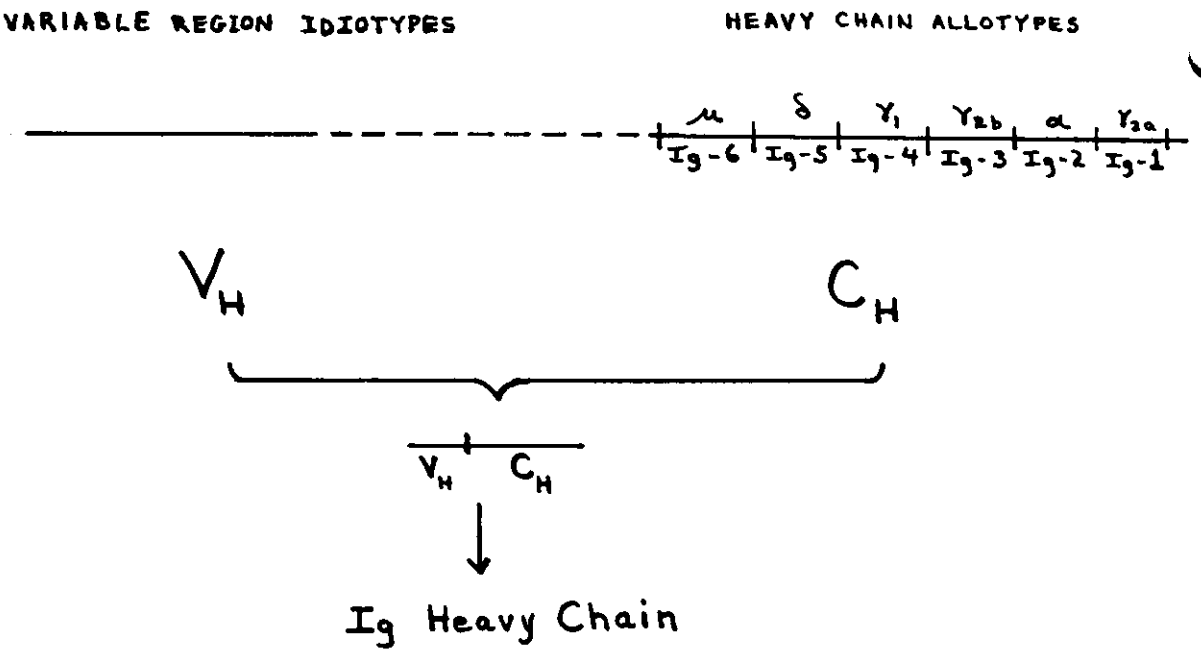

Figure 9. Immunoglobulin H-chain chromosome region. The order of the loci controlling the constant (Fc) portion of the H chain is unknown.

with anti-δ Ig-5b, and (2) ^{125}I-surface-labeled material precipitated with anti-Ig-5 and run in SDS-urea-PAGE under reducing conditions shows a band which runs with a mobility slightly greater than that of μ chain (apparent m.w. = 65,000).

In preliminary experiments using anti-allotype and heterologous antisera followed by FACS analysis, greater than 90% of splenic B cells (Ig-bearing) from adult mice were found to carry both IgM and IgD. A similar proportion of mesenteric lymph node B cells also carry both molecules. Studies of cocapping with the same reagents used above (anti-Ig-5b with goat anti-μ and anti-Ig-6b with rabbit anti-δ) on mesenteric lymph node cells confirmed that the IgM and IgD determinants detected are on different molecules which cap (redistribute) independently. Capping studies cited here were done in collaboration with Drs. E. Abney, J. Kearney, A. Lawton, M. Cooper and M. Parkhouse.

Allelic Exclusion for IgM and IgD Allotypes

Since roughly half the B cells in a Ig^a/Ig^b heterozygote stain with anti-Ig-6a or anti-Ig-6b and nearly all the B cells stain with goat anti-μ, the IgM-bearing cells appear to express only one of the parental alleles at the Ig-6 locus. Similar data were obtained staining heterozygotes for Ig-5 allotypes. These data suggest that both IgM and IgD, like IgA and IgG, are allelically excluded.

Staining heterozygotes for both Ig-5a and Ig-6b allotypes, using a single antiserum containing antibody to each (or with anti-Ig-5b and anti-Ig-6b), did not significantly increase the proportion of stained cells above that obtained by staining with either reagent alone (see Fig. 10). This suggests that a given cell expresses either *a* or *b* allotypes. Therefore, since virtually all B cells have both IgM and IgD allotypes on the surface, what we termed "allelic exclusion" above should probably more appropriately be called "haplotype exclusion," to semantically correct for the coordinated expression of the Ig-5 and Ig-6 alleles found on one of the parental chromosomes. In other words, if a single cell is either Ig-5a and Ig-6a or Ig-5b and Ig-6b, then allelic exclusion in this case means commitment to one parental chromosome (haplotype) or the other. Whether this haplotype commitment holds when the cell switches to an IgG memory cell (if it does switch) remains to be determined.

Haplotype exclusion in IgD- and IgM-bearing cells will add some new constraints to hypotheses on the mechanism of gene expression in immunoglobulin H-chain synthesis. IgG and IgA C_H and V_H regions show haplotype exclusion; that is, in a given IgG or IgA antibody molecule, the C_H and V_H almost always come from the same chromosome. A single intrachromosomal loop and excision could be responsible for this process. In a cell where two H-chain genes are expressed, such a simple mechanism is difficult to envision, especially since the same V_H region appears to be expressed both in IgM and IgD molecules on a given cell (Pernis et al. 1974). But with the data presented here emphasizing that both C_H genes expressed are on the same chromosome as the V_H gene, we may now have to become even more imaginative in devising a

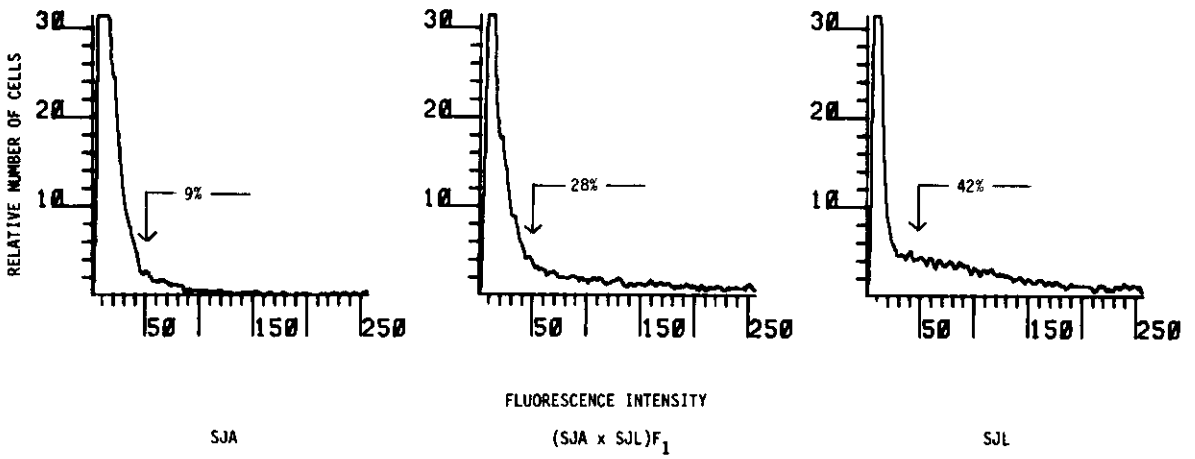

Figure 10. Allelic exclusions for cell-surface immunoglobulins. Cells were stained as described in the legend to Fig. 7; however, the antiserum used here was a mixture of anti-Ig-5b and anti-Ig-6b antibodies with approximately equal activity for each allotype. Percentage of cells showing greater than 50 units of fluorescence intensity for each type of spleen cell stained is indicated in each panel. Roughly half the number of B cells stain in the Ig^a/Ig^b heterozygotes as in the Ig^b homozygote. This suggests that both allotypes are allelically excluded and that heterozygous B cells express the same haplotype, e.g., Ig-5b and Ig-6b.

hypothesis to explain the mechanism of expression of immunoglobulin genes. For example, the V_H gene may be duplicated before or during translocation.

CONCLUDING REMARKS

The complex organization inherent in biological processes is displayed in all its natural beauty in the cellular interactions governing the various stages of B-cell differentiation to IgG AFC. As shown in Figure 11, even with drastic simplification, several independently regulated differentiational events involving interactions among at least three types of cells must be drawn to summarize the information we have presented in this report. But other types of cells should also be added to this rudimentary network; for example, in Figure 11 we have omitted IgM AFC and precursors of helper and suppressor T cells. In addition, the network should be branched at one of the early stages before the first cell committed to IgG (or IgA or IgE) class. At that point, a series of parallel networks should issue, one for each class, each network essentially similar to the one we have drawn. Again, at a stage even earlier, when variable-region commitment takes place, a multitude of branches must be drawn, one for each variable region alone. Although relatively few of these branches will mature all the way to IgG or other AFC, the amazing number of branch points and cell lineages that must exist in a single immunocompetent individual staggers the imagination.

T cells, as regulators of antibody production, somehow create order out of this massive potential confusion. The kinetics of immune responses and the order and frequency of the various H-chain classes produced remain relatively constant even

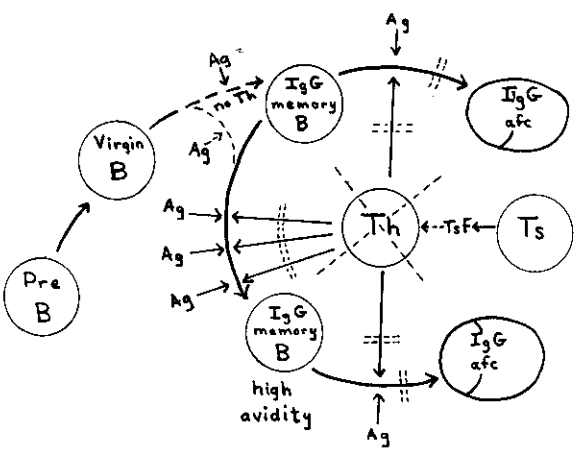

Figure 11. Rudimentary network for IgG production and avidity maturation.

over a wide range of dissimilar antigens. For those antigens or immunization protocols that deviate from this pattern, there is also a regularity of response which is T-cell regulated, at least in part (Gershon 1974).

The data we have discussed here offer some insights into these regulatory processes, although we are far from establishing definitive mechanisms. To summarize what we have shown, T_H appear to play a pivotal regulatory role, acting as the currency of the immune system, specialized in some cases for clonal branches expressing a given IgG class (or allotype), i.e., Ig-1b T_H. T_S, by removing T_H, serve as a kind of biological Fed (Federal Reserve Board), selectively regulating currency availability to control the AFC economy. Continuing this somewhat Marxist analogy, *I*-region loci, which are selectively expressed on the various lymphocytes and lymphocyte products involved in the network

(Murphy et al. 1976), seem to comprise the telecommunications system utilized for currency transfer and regulation. All of which leads us to suspect that development of some clever "bugging" devices or probes may be required to expose the inner machinations of the combine.

Acknowledgments

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REFERENCES

ABNEY, E. R., I. R. HUNTER and R. M. E. PARKHOUSE. 1976. Preparation and characterisation of an antiserum to the mouse candidate for immunoglobulin D. *Nature* **259:** 404.

CANTOR, H. and E. A. BOYSE. 1975a. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **145:** 1376.

———. 1975b. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. *J. Exp. Med.* **141:** 1390.

DIAMANTSTEIN, T. and E. BLITSTEIN-WILLINGER. 1974. T cell-independent development of B memory cells. *Eur. J. Immunol.* **4:** 830.

FELDMANN, M. 1972. Cell interactions in the immune response *in vitro*. V. Specific collaboration via complexes of antigen and thymus-derived cell immunoglobulin. *J. Exp. Med.* **136:** 737.

GERSHON, R. K. 1974. T cell control of antibody production. In *Contemporary topics in immunobiology* (ed. M. D. Cooper and N. L. Warner), vol. 3, p. 1. Plenum Press, New York.

GERSHON, R. K. and W. E. PAUL. 1971. Effect of thymus-derived lymphocytes on amount and affinity of antihapten antibody. *J. Immunol.* **106:** 872.

GODING, J. W., G. W. WARR and N. L. WARNER. 1976. Genetic polymorphism of IgD-like cell surface immunoglobulin in the mouse. *Proc. Nat. Acad. Sci.* **73:** 1305.

HERZENBERG, L. A. 1965. A chromosome region for $gamma_{2a}$ and $beta_{2a}$ globulin H chain isoantigens in the mouse. *Cold Spring Harbor Symp. Quant. Biol.* **29:** 455.

HERZENBERG, L. A., P. P. JONES and L. A. HERZENBERG. 1974. Mouse immunoglobulin allotypes: Characterization and use in cellular immunology. *Ann. Immunol.* (Inst. Pasteur) **125c:** 71.

HERZENBERG, L. A., H. O. MCDEVITT and L. A. HERZENBERG. 1968. Genetics of antibodies. *Annu. Rev. Genet.* **2:** 209.

HERZENBERG, L. A., K. OKUMURA and L. A. HERZENBERG. 1975a. Mechanism of allotype suppression. In *Symposium on suppressor cells in immunology* (ed. S. K. Singhal and N. R. Sinclair), p. 93. University of Western Ontario, Canada.

HERZENBERG, L. A., K. OKUMURA and C. M. METZLER. 1975b. Regulation of immunoglobulin and antibody production by allotype suppressor T cells in mice. *Transplant. Rev.* **27:** 57.

HERZENBERG, L. A., K. OKUMURA, H. CANTOR, V. L. SATO, F. W. SHEN, E. A. BOYSE and L. A. HERZENBERG. 1976. T cell regulation of antibody responses: Demonstration of allotype specific helper T cells and their specific removal by suppressor T cells. *J. Exp. Med.* **144:** 330.

HUBER, B., O. DEVINSKY, R. K. GERSHON and H. CANTOR. 1976. Cell-mediated immunity: Delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses. *J. Exp. Med.* **143:** 1534.

JERNE, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol.* (Inst. Pasteur) **125c:** 373.

JULIUS, M. H., E. SIMPSON and L. A. HERZENBERG. 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* **3:** 645.

KEARNEY, J. F., M. D. COOPER and A. R. LAWTON. 1976. B lymphocyte differentiation induced by lipopolysaccharide. III. Suppression of B cell maturation by anti-mouse immunoglobulin antibodies. *J. Immunol.* **116:** 671.

KISHIMOTO, T. and K. ISHIZAKA. 1973. Regulation of antibody response *in vitro*. VI. Carrier-specific helper cells for IgG and IgE antibody response. *J. Immunol.* **111:** 720.

LAWTON, A. R., III and M. D. COOPER. 1974. Modification of B lymphocyte differentiation by anti-immunoglobulin. In *Contemporary topics in immunobiology* (ed. M. D. Cooper and N. L. Warner), vol. 3, p. 193. Plenum Press, New York.

MCDEVITT, H. O., T. L. DELOVITCH, J. L. PRESS and D. B. MURPHY. 1976. Genetic and functional analysis of the Ia antigens: Their possible role in regulating the immune response. *Transplant. Rev.* **30:** 197.

MCKENZIE, I. F. C. 1975. Ly 4.2: A cell membrane alloantigen of murine B lymphocytes. II. Functional studies. *J. Immunol.* **114:** 856.

MURPHY, D. B., L. A. HERZENBERG, K. OKUMURA, L. A. HERZENBERG and H. O. MCDEVITT. 1976. A new *I* subregion *(I-J)* marked by a locus *(Ia-4)* controlling surface determinants on suppressor T lymphocytes. *J. Exp. Med.* (in press).

OKUMURA, K. L. A. HERZENBERG, D. B. MURPHY, H. O. MCDEVITT and L. A. HERZENBERG. 1976a. Selective expression of *H-2* (*I*-region) loci controlling determinants on helper and suppressor T lymphocytes. *J. Exp. Med.* **144:** 685.

OKUMURA, K., M. H. JULIUS, T. TSU, L. A. HERZENBERG and L. A. HERZENBERG. 1976b. Demonstration that IgG memory is carried by IgG bearing cells. *Eur. J. Immunol.* **6:** 467.

OKUMURA, K., C. M. METZLER, T. T. TSU, L. A. HERZENBERG and L. A. HERZENBERG. 1976c. Two stages of B cell memory development with different T cell requirements. *J. Exp. Med.* **144:** 345.

OWEN, J. J. T., M. D. COOPER and M. C. RAFF. 1974. *In vitro* generation of B lymphocytes in mouse foetal liver, a mammalian "bursa equivalent." *Nature* **249:** 361.

PARISH, C. R. 1975. Separation of functional analysis of subpopulations of lymphocytes bearing complement and Fc receptors. *Transplant. Rev.* **25:** 98.

PERNIS, B., J. C. BROUET and M. SELIGMANN. 1974. IgD and IgM on the membrane of lymphoid cells in macroglobulinemia. Evidence for identity of membrane IgD and IgM antibody activity in a case with anti-IgG receptors. *Eur. J. Immunol.* **4:** 776.

PERNIS, B., L. FORNI and L. AMANTE. 1971. Immunoglobulins as cell receptors. *Ann. N.Y. Acad. Sci.* **190:** 420.

ROELANTS, G. E. and B. A. ASKONAS. 1972. Immuno-

logical B memory in thymus deprived mice. *Nature New Biol.* **239:** 63.

ROWE, D. S., K. HUG, W. P. FAULK, J. N. MCCORMICK and H. GERBER. 1973. IgD on the surface of peripheral blood lymphocytes of the human newborn. *Nature New Biol.* **242:** 155.

SCHRADER, J. W. 1975. The role of T cells in IgG production; thymus-dependent antigens induce B cell memory in the absence of T cells. *J. Immunol.* **114:** 1665.

STOUT, R. D., S. D. WAKSAL and L. A. HERZENBERG. 1976. The Fc receptor on thymus-derived lymphocytes. III. Mixed lymphocyte reactivity and cell-mediated lympholytic activity of Fc^- and Fc^+ T lymphocytes. *J. Exp. Med.* **144:** 54.

STOUT, R. D., M. YUTOKU, A. GROSSBERG, D. PRESSMAN and L. A. HERZENBERG. 1975. A surface membrane determinant shared by subpopulations of thymocytes and B lymphocytes. *J. Immunol.* **115:** 508.

TADA, T. and M. TANIGUCHI. 1976. Characterization of the antigen-specific suppressive T cell factor with special reference to the expression of *I* region genes. In *The role of the products of the histocompatibility gene complex in immune responses* (ed. D. H. Katz and B. Benacerraf), p. 513. Academic Press, New York.

TADA, T., M. TANIGUCHI and C. S. DAVID. 1976. Properties of the antigen-specific suppressive T cell factor in the regulation of antibody response in the mouse. IV. Special subregion assignment of the gene(s) which codes for the suppressive T cell factor in the *H-2* histocompatibility complex. *J. Exp. Med.* **144:** 713.

YUTOKU, M., A. L. GROSSBERG, R. STOUT, L. A. HERZENBERG and D. PRESSMAN. 1976. Further studies on Th-B, a cell surface determinant present on mouse B cells, plasma cells and immature thymocytes. *J. Immunol.* (in press).