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Molecular, Cellular and Systemic Mechanisms for Regulating IgCH Expression

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INTRODUCTION

IgCH expression changes dramatically at several points along the antigen-triggered developmental pathway from "virgin" B to mature IgG-secreting (plasma) cells. IgD and IgM receptors are present on the virgin B cell and are still expressed on "early" memory cells. But the expression of these receptors terminates when early memory cells are triggered to differentiate to the "mature" memory cells (present in animals primed with hapten-carrier conjugates on alum or in other adjuvants). The expression of IgG receptors, in contrast, apparently begins when virgin cells differentiate into early memory cells and it continues thereafter. Thus, by the time B cells reach the mature memory stage, they have lost IgD and IgM and, instead, carry surface IgG molecules that indicate the antibody-secretion committment of the cell (see Figure 1 and Herzenberg et al. 1980a, Black et al. 1977, 1980, Okumura et al. 1976, Coffman & Cohen 1977, Abney et al. 1978).

At the molecular level, shifts in IgCH expression are regulated by mechanisms that enable or prevent genetic rearrangements and changes in RNA processing at key points during B cell development. At the cellular level, such shifts are controlled (triggered) by receptor-antigen interactions and by signals from regulatory cells that encourage or discourage B cell differentiation (e.g., Black et al. 1980, Okumura et al. 1976a). Operating together, these mechanisms thus define the IgCH isotype expressed by an individual memory cell and the

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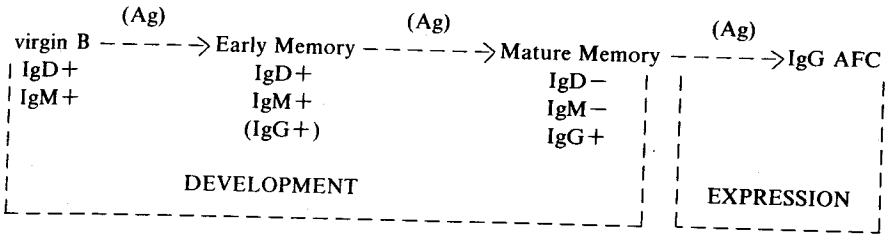
Figure 1. Schematic view of the memory B cell development pathway.



* Mechanisms that regulate memory B cell development control the potential for IgG antibody production.

* Mechanisms that regulate memory B cell expression control which and how many of the memory B cells present in a given animal actually give rise to AFC.

proportional representation of cells carrying each of the IgG isotypes in memory populations generated in response to antigenic stimulation.

The frequencies of memory B cells committed to producing various isotype or allotype responses to a given epitope, however, do not necessarily define the

TABLE I

Allotype-suppression impairs Igh-1b memory B cell expression rather than development

	DNP-KLH Primed Donors§		Adoptive IgG Anti-DNP Responses* (from splenic B cells+KLH T)			
Status	Age when primed	Igh-1b in situ†	Igh-1a	Igh-1b	Igh-4a	Igh-4b
Control	8 wk	normal	90	80	250	280
Suppressed	8 wk	no	93	73	270	260
Suppressed	>5 mo	no	130	170	nd	nd

§ SJL×BALB/C donors primed with 100 μg DNP-KLH on alum >4 weeks prior to transfer; allotype-suppressed mice exposed to maternal antibodies to the paternal Igh-1b (IgG2a) allotype.

* B cells remaining from 10^7 donor spleen cells treated with monoclonal anti-Thy-1.2 and complement (to deplete T cells) were mixed with syngeneic carrier-primed T cells (2.5×10^6 nylon-passed T cells from non-suppressed KLH-primed mice) and transferred to irradiated BALB/c mice (650 Rad, 18 h previously); Recipients were immunized with 1 μg aqueous DNP-KLH at time of transfer; serum anti-DNP antibody levels (μg/ml) were measured by RIA 2 weeks after transfer. Nylon-passed T cells transferred without B cells produced <5 μg/ml anti-DNP antibody of each allotype.

† Igh-1b serum levels <10 μg/ml; occasional very low and transient *in situ* Igh-1 anti-DNP responses produced; all other *in situ* IgG responses normal.

IgCH isotype expression (representation) in antibody responses to that epitope. That is, antigenic stimulation (with T-dependent antigens) that generates optimal memory B cell populations does not always stimulate *in situ* primary antibody responses. Depending on the immunization protocol, such stimulation can instead induce the (recently recognized) "epitope-specific" regulatory system to suppress the expression (expansion and terminal differentiation) of newly-generated or pre-existent memory cells. Furthermore, it can induce specific suppression for the expression of individual memory cells committed to producing antibodies with a particular IgCH constant-region and anti-epitope combining-site (Herzenberg & Tokuhisa 1982, Herzenberg et al. 1980b, 1981, 1982a–c).

This epitope-specific system essentially determines which and how many of the memory B cells present in an animal give rise to antibody secreting cells (afc) under a given set of stimulatory conditions. Thus mechanisms that regulate IgH gene expression in B cells control the potential for producing various antibodies; however, mechanisms that selectively regulate the actuation of this potential also exist and play a key role in defining the characteristics of antibody responses (magnitude, specificity, affinity and IgCH isotype/allotype representation) (Herzenberg et al. 1982a).

IgH-RESTRICTED REGULATION OF MEMORY B CELL EXPRESSION

In our experience, most of the differences in IgCH representation in *in situ* antibody responses measured following immunization with the kinds of antigens and protocols commonly used to study B cell memory reflect these latter regulatory influences on the expression (rather than the development) of memory B cells. That is, the failure of a given *in situ* antibody response rarely turns out to be due to the absence of memory B cells capable of giving rise to that response. Usually, such memory B cells are present and demonstrable in adoptive assays but fail to give rise to *in situ* responses because their expression is specifically suppressed.

Memory development in allotype-suppressed mice, for example, is somewhat impaired in that a higher proportion of the allotype-committed memory cells tend to remain at the early memory stage; however, by and large, allotype-suppressed mice have ample populations even though they do not produce allotype-marked antibody responses when the allotype-suppression suppressor T cell mechanism is active (see Table 1) (Jacobson et al. 1972, 1981, Okumura et al. 1976b, Black et al. 1980, Tokuhisa et al. 1980, 1981, Herzenberg & Herzenberg 1974, Herzenberg et al. 1976, 1982c). Similarly, idiotype-suppressed mice (at least in some systems) have normal memory populations even though they do not produce antibody responses containing the suppressed idiotype (Eichman et al. 1978).

RNA transcription and processing

The two variants in which we have thus far failed to detect rearrangements are both recently isolated lines that switched to producing IgE antibodies. One of these is derived from a parent producing normal IgG2a antibodies while the other is derived from a parent producing a "short-chain IgG2a" molecule (lacking most or all of the CH1 domain). Southern analyses of the DNA from these parents and variants (with 10 restriction enzymes) have not revealed a restriction enzyme recognition sequence that shows a J gene segment context which differs among these four cell lines.

The failure to detect rearrangements in these IgE-producing variants may be due merely to our choice of restriction enzymes. That is, analyses of digests with additional restriction enzymes (currently in progress) may yet reveal a J gene DNA segment difference. We are intrigued, however, by the possibility that the regulation of immunoglobulin gene expression in these cell lines is controlled at another level, perhaps via RNA processesing. This idea is supported by the unique pattern of changes in isotype production we observed in these variants during the early stages of the selection process. In both cases, we isolated transient IgG2a/IgE double-producing clones that quickly segregated to production of one or the other immunoglobulin isotype upon further cloning. (We have not as yet biochemically analyzed the IgG2a products produced by the clones that segregated to produce the parental type).

These findings may be attributable to the production of an extended (VDJ-IgG2a-IgE) transcript that tends to be processed initially to permit the expression of both CH genes but is later processed to permit the expression of one or the other. If so, then we would expect to find a long nuclear RNA transcript in these cells which contains sequences of both the IgG2a and IgE genes. We are presently testing this hypothesis by doing Northern analyses of nuclear RNA from these cell lines (although we must admit that some valid objections can be raised concerning the likelihood that RNA processing differences account for the findings with these variants).

DNA methylation

Undermethylation of "active" genes seems to be commonplace in eukaryotic genomes (Razin & Friedman 1981). This generalization apparently holds for switch variants since, in the four variants we have examined thus far, the transcribed CH genes are the only undermethylated CH genes in each case. Furthermore, the controlling element with respect to undermethylation apparently moves with the VDJ segment. For example, when a cell switches from an IgG1-producer to an IgG2b-producer, the IgG2b gene sites become undermethylated. This evidence demonstrates directly that rearrangement and switching in the variants tested is accompanied by a change in CH gene methylation such that under-methylation remains correlated with CH gene expression. Thus it suggests that genetic information in the VH gene complex (V-D-J and its associated flanking and intron sequences) controls the methylation of downstream CH gene(s) (Oi et al. unpublished observations).

This idea raises a variety of questions, some immediately amenable to test and others of more long-term but nonetheless cogent interest. What methylation pattern will we find, for example, in the IgE-producing variants mentioned above in which we have not been able to detect a VH rearrangement accompanying the switch from IgG2a production? Or, returning to the normal B cell subpopulation discussed earlier, what contrasts in methylation patterns will we find in mature cells expressing different levels of IgM and IgD. Similarly, what methylation patterns can be expected in immature B cells and germinal center cells that only express IgM receptors? (If data obtained with a tumor cell line producing both IgM and IgD is predictive, then the expressed IgM and IgD CH genes in mature B cells should be under-methylated (Rogers & Wall 1981); however, it is "anybody's guess" as to what the methylation status of the IgD gene is prior to its expression).

Attempting to predict normal cell behavior from studies with tumor cells or with the hybridoma variants we have isolated is, of course, a risky matter in any event; however, such studies are clearly useful for defining and refining hypotheses before more complex studies with normal populations are initiated. Thus we see a two-fold purpose to our work with switch-variants and other types of animal cell lines: first, such studies reveal the untrammeled potential of the animal genome; and, secondly, they offer insights which may prove useful in devising studies to clarify the workings of such cells in their natural habitat.

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TABLE II

Competent memory B cell populations in normal animals are not always expressed in situ

Immunization(s)*		IgG2a anti-DNP Antibody in Serum	
KLH	DNP-KLH	In Situ Primary μg/ml (affinity)	Adoptive Secondary (donor B cells+CTh) μg/ml (affinity)
alum	aqueous†	5 (<1)	50 (8)
alum	alum†	5 (<1)	73 (8)
alum	aqueous	3 (<1)	18 (<1)
alum	alum	35 (5)	73 (10)

* 100 μg indicated antigen; 4 wks between KLH and DNP-KLH; Cell tranfers and response measurements as in Table I. Data shown for (BALB/C×SJL)F1 mice are representative of data from similar studies in BALB/C.

† Sequential carrier/hapten-carrier immunization induces specific suppression for *in situ* antibody responses to the "new" hapten on the carrier (see text).

Our recent studies with normal animals also demonstrate that memory B cell development is necessary but not sufficient to assure IgG antibody production.

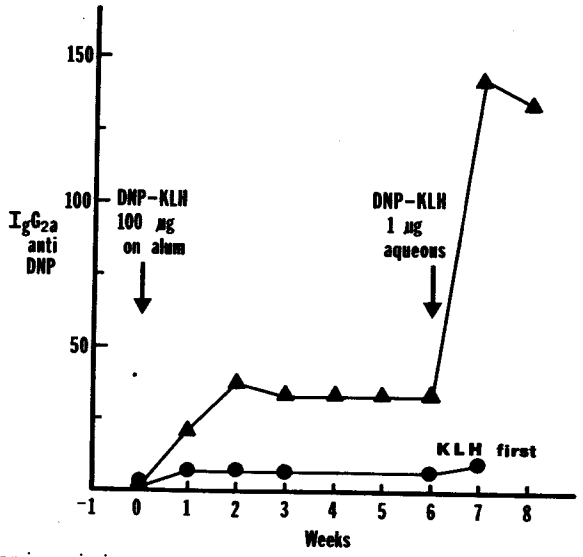

Figure 2. Carrier-priming prevents subsequent hapten-carrier stimulation of anti-hapten antibody responses. Data for Igh-1a (IgG2a) allotype anti-DNP responses shown are comparable to Igh-1b, IgG2b and IgG3 anti-DNP responses in the same (BALB/C×SJL)F1 allotype heterozygotes. IgG1 responses are also comparable in animals stimulated once with DNP-KLH; however, IgG1 responses in some KLH/DNP-KLH/DNP-KLH stimulated animals are equivalent to normal secondary anti-DNP responses levels (e.g., see Figure 5). For protocol and assay details, see legend for Figure 3.

TABLE III

The epitope-specific system selectively regulates isotype representation in antibody responses

Immunizations*			Relative Anti-DNP Levels in Serum†				
			IgM	IgG1	IgG3	IgG2b	IgG2a
–	DK		1	1	1	1	1
K	DK		2	0.5	0.3	0.4	0.2
–	DK	DK	3	10	8	13	7
K	DK	DK	4	8	2	1	0.3

* K=KLH; DK=DNP-KLH; 100 μg each antigen on alum at 4 wk intervals; 10 or more (BALB/c×SJL)F1 mice per group;

† Mean responses (normalized to primary response to DNP-KLH) measured by RIA 2 weeks after last immunization (3). See Figure 1 or Table II for representative (absolute) IgG1 and IgG2a responses in individual animals. IgG1 responses in K/DK/DK animals were broadly distributed (see Figure 2); other responses were more tightly grouped around the mean response shown.

That is: immunization with a "priming dose" of a hapten-carrier conjugate (e.g., 100 μg on alum) invariably induces the appearance of mature anti-hapten memory B cell populations with similar activity in adoptive assays; however, the *in situ* IgG anti-hapten responses that follow this immunization vary markedly

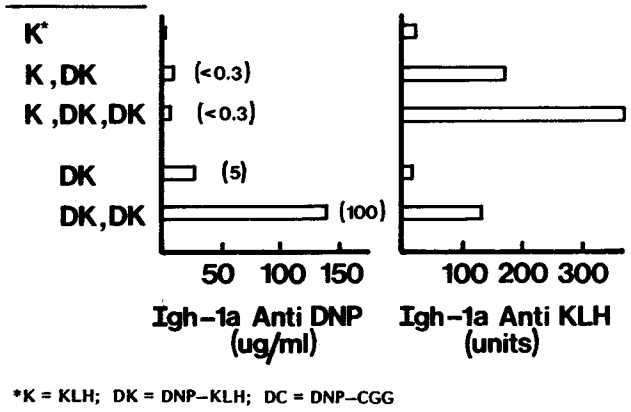

Figure 3. Carrier/hapten-carrier immunization induces epitope-specific suppression for IgG anti-hapten responses: secondary stimulation with DNP on the same carrier. Animals were immunized with 100 μg of the indicated antigens (on alum) at 6-week intervals. Units of anti-carrier antibody are calculated relative to a standard adoptive secondary response serum (to the appropriate carrier). Data in parentheses after anti-DNP response "bars" show the average Ka ($M^{-1}\times10^6$) for the response. Mean antibody levels shown, measured 2 weeks after stimulation or restimulation (by RIA) in individual serum samples, are representative of serum antibody levels measured weekly thereafter (for at least 4 weeks).

producing clones yielded downstream (IgE-producing) variants that do not appear to have arisen from typical rearrangements. That is, we have not been able to distinguish these from the parent clones by restriction mapping although we have examined digests with 10 restriction enzymes. We are not yet ready to rule out rearrangement as accounting for these IgG2a to IgE switches on the basis of this (negative) evidence; however, we have begun exploring alternative mechanisms that could account for the switch in these variants (see discussion of DNA methylation below).

CHARACTERIZATION OF THE GENETIC MECHANISMS UNDERLYING IgCH SWITCHES IN HYBRIDOMA VARIANTS

The following sections summarize our studies analyzing the variant lines we have obtained for three kinds of mechanisms that could account for switches in IgCH expression: rearrangements, somatic crossing-over or other chromosomal changes; methylation or other DNA modifications affecting transcription; and, differential splicing of primary transcripts or other mechanisms affecting translation.

Intra-chromosomal rearrangements

As indicated above, the switch in IgCH expression in some of the variants we have obtained is due to IgH chromosomal rearrangements in which the VDJ gene segment is juxtaposed to a "new" CH gene that then encodes exclusively for the immunoglobulins produced by the cell. We base this conclusion on Southern hybridization analysis of DNA from the variant cell lines using a DNA probe which includes both the J3 and J4 genes (Newell et al. 1980). (This probe is broadly useful for identifying rearrangements in switch variants because a J4 segment is retained (not deleted) in the VDJ gene segment used by these cell lines regardless of which J gene actually is used in the VDJ gene expressed.) Thus, by following the fate of the J4 gene segment in each of the newly-selected variants, we have shown that the switches in four of the six variants studied thus far are clearly accompanied by the rearrangement of at least one DNA restriction fragment that hybridizes with the (J4) probe.

The demonstration of these rearrangements implies the deletion of the formerly utilized upstream gene; however, in attempting to directly demonstrate this deletion, we have been confronted by two related problems. First, the multiple copies of all of the immunoglobulin genes in the variant cells make it extremely difficult to know whether overlapping restriction fragments are being differentially affected by the switching phenomenon. Secondly, although the fusion parents of our most extensively analyzed variant family have different

IgH haplotypes, data from Southern hybridization analyses have not as yet revealed any restriction site polymorphisms between these haplotypes. Consequently, it is presently impossible to distinguish which gene copies are from the active (C3H.SW) chromosome.

To make matters worse, we have found differences between the rearrangements that occurred in a pair of variants that each switched (independently) from IgG1 to IgG2b production. In one, we clearly see an IgG1 restriction fragment disappear in the IgG2b producer. However, in the other, the IgG2b producer gains a novel IgG1 restriction fragment. What this novel IgG1 context represents can be understood only after cloning and perhaps sequencing this new restriction fragment; however, these findings certainly appear inconsistent with a simple deletion model for class switching.

Inter(?)-chromosomal mechanisms

"Back-switch" variants pose another kind of mechanistic puzzle in that they appear to be due to rearrangements in which the VH is juxtaposed to an upstream CH gene which, in principle, should have been excised from the chromosome during an earlier rearrangement. We have been trying to determine whether the "new" CH gene is drawn from an IgCH segment duplicated downstream on the active chromosome or from an IgCH region on a different (inactive) chromosome. Unfortunately, these variants have all been selected from a parental clone derived from the fusion of allotypically (IgH) similar cells (C3H.SW spleen cells and NS-1 myeloma cells). Thus allotype markers on the immunoglobulin produced by the variant can't be used to identify the source of the CH gene being expressed; and, for somewhat similar reasons, recombinant DNA analyses have yielded ambiguous data on this point (both in our laboratory and in collaborative studies with Dr. Tasuku Honjo, Osaka University).

We are now attempting to resolve this issue by selecting back-switch variants in a variant family derived from a potentially more informative initial fusion. The active chromosome in this family codes for allotypically-marked CH structures that are all easily distinguishable from the BALB/C allotypes coded for by CH genes on NS-1 chromosomes. Thus far we have succeeded in isolating several "cross-allotype" back-switch variants from an IgG2a-producing clone in the family; however, all have reverted to the parental IgG2a (Igh-1b allotype) phenotype after a short period in culture. This instability introduces another intriguing area for study but at present contributes little towards identification of the origin of the CH gene in stable back-switch variants such as those obtained previously. Nevertheless, because a stable cross-allotype back-switch variant would provide definitive evidence on this point, we are currently continuing attempts to select such variants (Oi, unpublished observations).

according to whether the hapten-carrier conjugate is introduced under conditions that permit the induction of support or suppression for the various

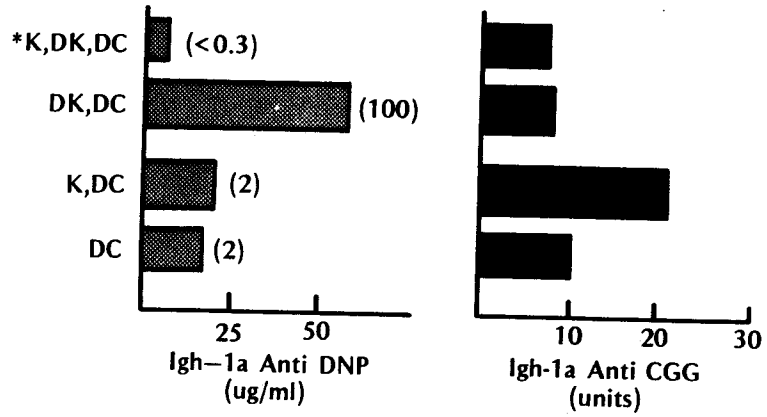

Figure 4. Carrier/hapten-carrier immunization induces epitope-specific suppression for IgG anti-hapten responses: secondary stimulation with DNP on a different carrier. For protocol and assay details, see legend for Figure 3.

TABLE IV

Individual isotype responses are independently regulated by the epitope-specific system

Animal number	Immunizations			IgG Anti-DNP Response			
				IgG3	IgG1	IgG2b	IgG2a
1	K	DK	DK	10	260	33	<6
2	K	DK	DK	15	360	48	<6
3	K	DK	DK	7	130	15	<6
4	K	DK	DK	23	680	120	40
5	K	DK*	DK	5	200	62	16
6	K	DK	DK	12	310	55	15
7	–	DK	DK	14	260	120	170
8	–	DK	DK	30	420	170	220
9	–	DK	DK	50	150	93	160
10	–	DK	DK	55	400	100	240
11	–	DK	DK	38	310	120	190

* Responses shown are from animals in a single experiment in which the KLH preparation used primed poorly for suppression induction. Generally, carrier/hapten-carrier immunized animals require three DNP-KLH stimulations (100 μg on alum each time) to reach this degree of responsiveness. For protocol and assay details, see legend for Figure 3. IgG1 and IgG2a responses reported as μg/ml serum; IgG3 and IgG2b reported as estimated μg/ml serum (based on comparisons of counts bound in RIA rather than on binding with direct standards).

components of the "normal" anti-hapten response (Herzenberg et al. 1980, 1982a).

Presentation of the hapten-carrier conjugate *de novo,* for example, permits production of typical primary and secondary anti-hapten responses in which all IgG isotypes are well represented. Presentation of the same hapten-carrier conjugate to animals primed previously with the carrier protein but "naive" with

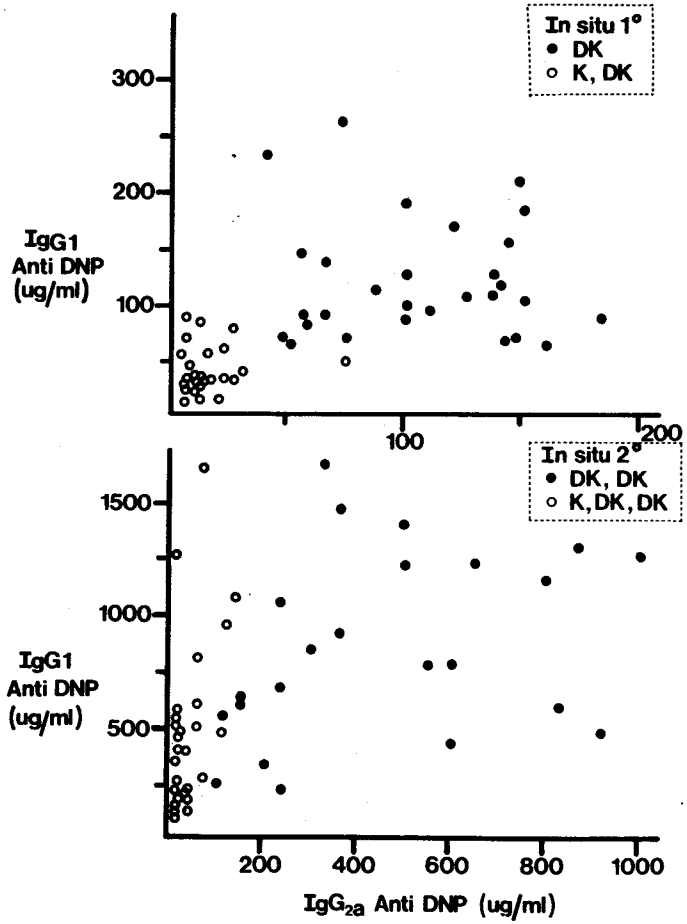

Figure 5. IgG1 responses escape suppression more easily than IgG2a responses. Axes indicate μg/ml anti-DNP antibody of the specified isotype in serum samples from individual animals. Animals were primed with 100 μg DNP-KLH (DK) or KLH (K) on alum and then stimulated 6 weeks later with DNP-KLH (again 100 μg on alum). Antibody levels shown, measured 2 weeks after stimulation or restimulation, are representative of levels measured weekly thereafter (at least 4 weeks). Responses from the (BALB/c×SJL)F1 hybrids used for these experiments were similar to responses obtained with BALB/ć and BAB/14 mice.

ing IgCH contributions to the physical and functional properties of the antibody molecule (Oi 1982, abstract). Thus we used a hybridoma line producing IgG1 anti-dansyl antibodies for the parent of the (first) switch-variant family we isolated (see Figure 15) and then used the antibodies produced by the parent and variant lines to compare the functional properties of different constant regions (in association with the same combining-site). The results of these studies (Oi et al. 1981), summarized in Table VIII, define clearcut complement fixation differences amongst the isotypes: IgE antibodies do not fix complement; IgG1 antibodies are relatively ineffective in this regard; and intact IgG2 antibodies show strong complement fixation (IgG2b>IgG2a). In addition, these data

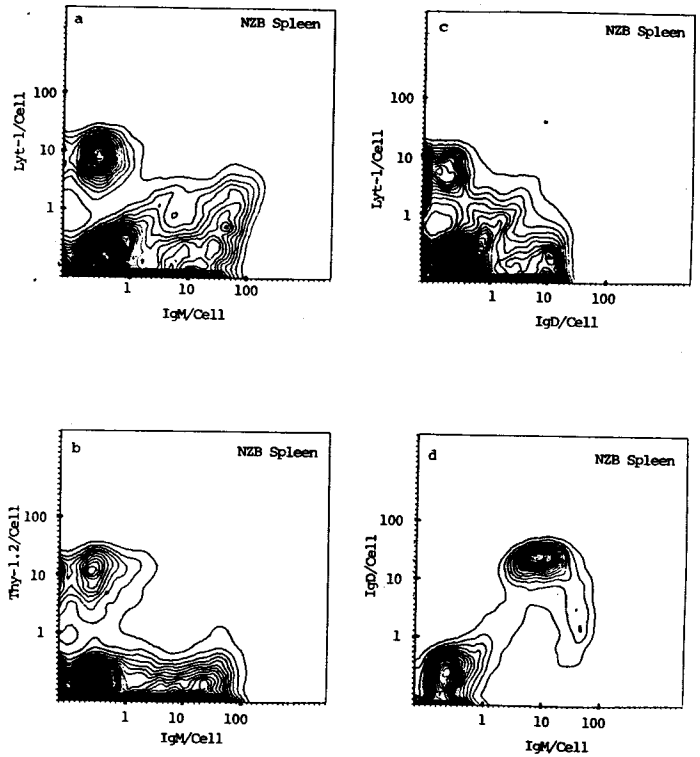

Figure 14. Lyt-1 determinants are found on population III. The IgM-Lyt-1 staining (a) shows that IgM-bright cells have small amounts of a surface determinant that react with monoclonal anti-Lyt-1, while the IgD-Lyt-1 staining (c) shows that Lyt-1-staining cells are IgD-dull. Together, these stains define population III (high IgM, low IgD). Data from NZB mice are shown since this strain has relatively large numbers of cells in population III (b). The Thy-1/IgM staining is presented as a specificity control to show that a monoclonal antibody of the same isotype (rat IgG2) does not stain population III.

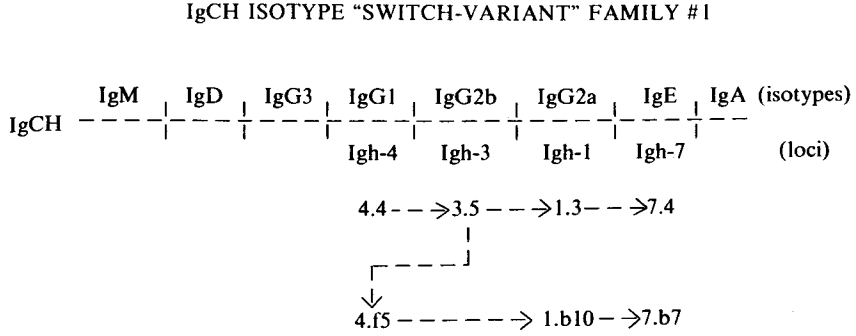

Figure 15. IgCH isotype "switch-variant family 1. Variants are columnated according to isotype produced.

demonstrate an intriguing correlation between the segmental flexibility of an antibody molecule and its complement fixing potential.

At the genetic level, the hybridoma variants we selected were initially quite similar to the plasmacytoma variants obtained and selected by Rajewsky and co-workers. We also obtained downstream switches and upstream "back-switches". Furthermore, the downstream switches are clearly identifiable as typical rearrangements in which the VH gene complex is juxtaposed to the next CH gene on the active chromosome (since the restriction maps from the parental and variant lines are distinguished from one another by the disappearance and appearance of the relevant restriction fragments).

In our most recent variant selections with this family, however, two IgG2a-

TABLE VIII

*Characteristics of antibodies from switch-variant family # 1 (anti-dansyl combining site)**

Parent	Variant	IgH Isotype	Complement Fixation	Segmental Flexibility
4.4		IgG1	+	rigid
4.4	3.5	IgG2b	+++	+++
3.5	1.3	IgG2a	++	++
1.3	7.4	IgE	no	rigid
3.5	4.f5	IgG1	+	rigid
4.f5	1.b10	IgG2a (no CH1)	++**	rigid (?)
1.b10	7.b7	IgE	no	rigid

* affinity (Ka M-1) >10**8.

** higher (10%) complement fixation without antigen.

respect to the hapten, however, usually results in the specific suppression of all of the potential primary IgG anti-hapten responses and the persistent suppression of most of these responses (see Figures 2 through 6).

The regulatory mechanism that mediates the suppression induced by this carrier/hapten-carrier immunization sequence is epitope-specific and Igh-restricted. That is, once induced to suppress antibody responses to an epitope (hapten) on a carrier protein, it suppresses responses to that epitope presented on an unrelated carrier molecule and does not interfere with primary or

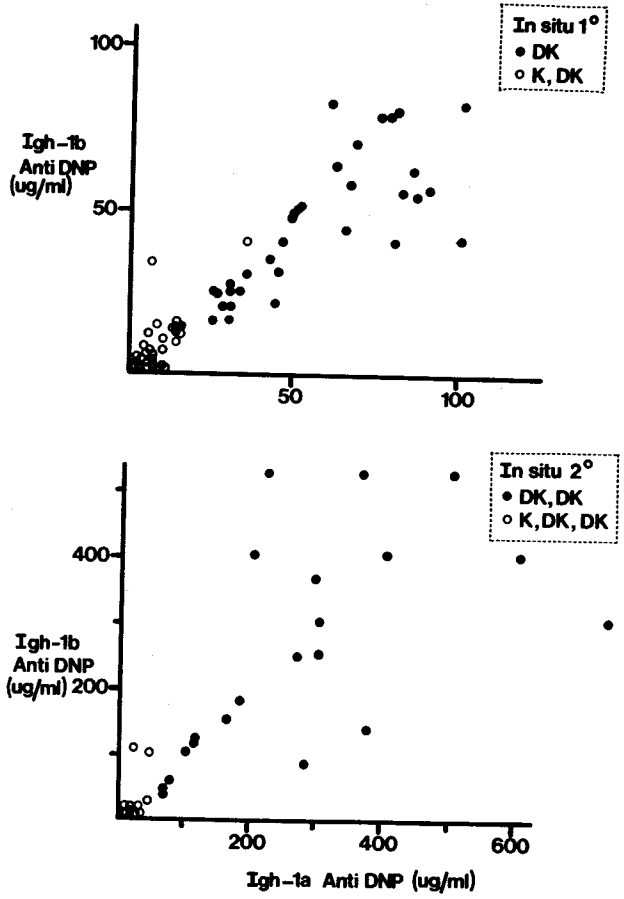

Figure 6. Igh-1a and Igh-1b (IgG2a allotypes) anti-DNP responses tend to be concordantly regulated in normal (not allotype-suppressed) animals. Axes indicate μg/ml anti-DNP antibody of the specified allotype in serum samples from individual animals. (BALB/c×SL)F1 hybrids were used for these experiments. (For protocol and assay details, see legend for Figure 5).

TABLE V

*Selective suppression for Igh-1b anti-epitope responses (induced by the allotype-suppression mechanism)***

DNP-KLH Primed mice*	Second Antigen†	Igh-1 (IgG2a) Allotype Antibody Responses§ Anti-DNP (secondary) Igh-1a	Anti-DNP (secondary) Igh-1b	Anti-KLH (secondary) Igh-1a	Anti-KLH (secondary) Igh-1b	Anti-CGG (primary) Igh-1a	Anti-CGG (primary) Igh-1b
Group #1	DNP-KLH	130	<5	60	<5		
Group #1	DNP-CGG	260	10			14	14
Group #2	DNP-CGG	316	195			13	18
Control	DNP-KLH	127	110	25	40		

* Group #1: young (BALB/C×SJL)F1 mice primed while allotype suppression is active (no Igh-1b primary antibody responses to DNP-KLH, specific suppression induced for subsequent Igh-1b responses to DNP-KLH epitopes). Group #2: mice primed after the onset of remission from allotype suppression (normal Igh-1b primary responses to DNP-KLH, no epitope-specific suppression induced). Control protocol: normal syngenic age-matched control mice primed with same antigen dose (100 μg DNP-KLH on alum). All groups induce similar (normal) Igh-1b memory for DNP-KLH epitopes (e.g., see Table I).

† Second immunization: all allotype-suppressed mice now in remission (suppression for Igh-1b responses to DNP-KLH epitopes persists if induced previously; Igh-1b responses to newly-introduced CGG epitopes are normal, other IgG responses to DNP-KLH and DNP-CGG epitopes also normal).

§ Serum antibody levels tested by (RIA) 14 days after immunization with indicated antigen. Anti-CGG and anti-KLH activity (units) expressed relative to an appropriate secondary response antiserum. Anti-DNP responses expressed as μg/ml serum.

** Studies summarized from Herzenberg et al. 1982c.

secondary antibody responses to other epitopes on either carrier. Furthermore, although it can suppress all IgG responses to the epitope, it often selectively suppresses individual isotype or allotype anti-epitope responses.

That is: IgG1 antibody responses are more resistant to suppression than other IgG responses, including IgG3 (see Table III). IgG3 responses, however, tend to be more resistant to suppression than IgG2a and IgG2b. Thus when suppression is induced initially under sub-optimal conditions or begins to wane following repeated stimulation with the hapten-carrier conjugate, animals tend to produce anti-hapten responses composed almost exclusively of IgG1 antibodies (Herzenberg et al. 1982a–c). Nevertheless, individuals in which suppression is weak occasionally produce exclusively IgG2a, IgG2b or IgG3 responses or, more frequently, produce IgG1 responses together with responses to one or another of the "more suppressible" isotypes (see Table IV and Figure 5).

We have also observed occasional (carrier/hapten-carrier) suppressed allotype heterozygotes that partially escape from suppression and produce single allotype anti-hapten responses (see Figure 6). Furthermore, we have induced

receptor expression imply differential function; could the differences in receptor expression reflect the operation of distinct DNA attenuator sequences associated with IgD and IgM; etc.). Hopefully, the answers to some of these questions will be obtained by examining the functional roles, lineage relationships and molecular regulatory mechanisms of sorted B cells in the subpopulations defined by the analytic parameters discussed here.

IgCH expression in hybridoma "switch" variants

Hybridomas, like plasmacytomas, have an active IgH chromosome on which a VDJ segment juxtaposed to an IgCH segment (VDJ rearrangement) defines the

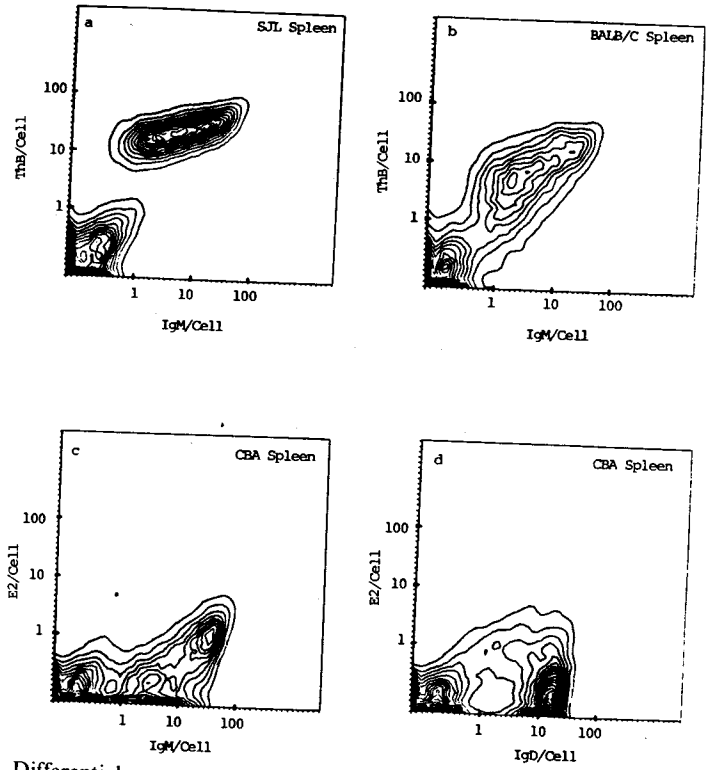

Figure 12. Differential representation of two non-Ig B cell surface markers on B cell subpopulation. Differences in ThB surface density on population I account for the major difference between mouse strains that carry the "high" and "low" alleles for ThB expression. SJL (a) is a high strain and BALB/c (b) is a low strain. The "E2" antigen (detected by a rat monoclonal antibody) is expressed only on populations II and III (high IgM, variable IgD), i.e., this marker is present on cells that have high but not low surface IgM density (c) and is found on cells that have either low or high surface IgD density (d).

Ig heavy chain structure of the secreted and cell-surface antibody molecules produced by the cell (Honjo et al. 1981). The "upstream" IgCH genes on this chromosome are deleted (by the rearrangement) while the "downstream" genes remain in their germ-line configuration. Thus, in principle, new rearrangements ("switches") that result in the expression of the downstream IgCH genes should be possible in established hybridomas and plasmacytomas. In addition, because these kinds of neo-plastic cells generally have at least one additional (inactive) IgH chromosome, inter-chromosomal exchanges that result in the expression either of downstream or upstream switches in IgCH expression are also possible (at least in theory).

In practice, IgCH switches of any type appear to be extremely rare since, even when plasmacytomas are grown for years, they almost always continue to produce the same IgCH isotype they produced originally. Nevertheless, as Rajewsky and co-workers recently showed, rare spontaneous isotype "switch" variants do arise in cultured plasmacytoma lines and can be isolated by FACS sorting for (selecting) variant cells that express a "new" surface isotype. Furthermore, these investigators showed that new variants could be selected from established variant lines and thus a family of switch variants could be developed by sequential selections. Most of the variants obtained were explicable as the result of straightforward downstream switches to the next IgCH gene on the active chromosome; however, selections for "back-switch" variants that express IgCH genes presumably deleted from the active parental chromosome were also successful.

These findings encouraged our (successful) attempts to isolate switch variants from hybridoma cell lines producing anti-hapten antibodies useful for examin-

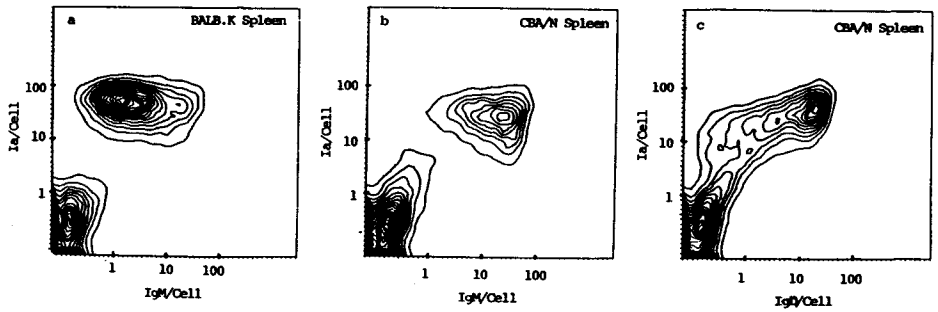

Figure 13. Ia expression is lower on population III. The IgM/Ia staining (a,b) shows that cells with the least Ia cells are IgM-bright, while the IgD/Ia staining (c) shows that cells with the least Ia cells are IgD-dull. Together, these stains define population III (high IgM, low IgD). Data for CBA/N (b,c) are more informative because population I is less dominant than in BALB/C.

specific suppression for Igh-1b (IgG2a allotype) antibody responses to all of the epitopes on DNP-KLH (by priming young allotype-suppressed mice with DNP-KLH prior to the onset of the characteristic mid-life remission from allotype suppression) (see Table V). These latter findings demonstrate clearly that the epitope-specific system can selectively regulate the production of individual allotype-marked antibody responses. Consequently, they suggest that this system normally regulates such responses independently but tends to maintain allotype concordancy for a given isotype unless immunization conditions dictate the specific suppression of one allotype rather than both (Herzenberg et al. 1982b).

Allotypic determinants, in fact, are almost always unique to a single isotype and thus identify both the isotype and the allotype of an antibody molecule

TABLE VI

The epitope-specific system is a general regulatory mechanism§

Variable Examined	Result Obtained
* Epitope	Suppression induced for DNP and TNP by carrier/hapten-carrier; suppression inducible for KLH epitopes by other protocols
* Carrier	KLH, CGG, Ovalbumin and TGAL all prime for carrier/hapten-carrier suppression induction; some genetic restrictions (e.g., KLH in A/J); 100 μg KLH aqueous, on alum or in CFA about equally effective; addition of B. pertussis antigen prevents suppression induction.
* Timing/Age	Suppression induction equally strong in adults of all ages; persists for up to 1 year once induced; 1 to 13 weeks (or more) between carrier and hapten-carrier equivalent.
* Genetic Control	Suppression induction impaired by non-MHC "carrier-function" genes, e.g., KLH/DNP-KLH suppression induction impaired in A/J and C57BL/10 (CGG/DNP-CGG OK in these strains); no impairment by MHC IR genes that impair IgC antibody responses to epitopes on certain carriers, e.g., TGAL/TNP-TGAL induces typical epitope-specific suppression for IgG anti-TNP in Ir-1a "non-responders" to TGAL.
* Mouse Strains	Carrier/hapten-carrier can induce suppression in BALB/C, BAB/14, SJL, SJA, C3H, C3H.SW, A/J, (SJL×BALB/C), C57BL/10 and C57BL/6 mice.
* Other Regulatory Systems	Epitope-specific system mediates suppression by carrier-specific suppressor T cells (CTS), e.g., KLH-specific CTs regulate IgG antibody by inducing epitope-specific suppression; allotype suppression induces epitope-specific suppression; Ir non-responsiveness may also be mediated by the epitope-specific system.

§ Studies summarized from Herzenberg & Tokuhisa 1982 and Herzenberg et al. 1982a–c.

(Herzenberg & Herzenberg 1978). Thus the isotype-restricted regulation that characterizes the epitope-specific suppression induced by carrier/hapten-carrier immunization can be explained both in allotype homozygotes and heterozygotes by the activity of Igh-restricted epitope-specific regulatory elements that recognize allotypic determinants and usually tend to operate concordantly for a given isotype in allotype heterozygotes.

Generality of epitope-specific regulation

Mechanisms that regulate isotype, allotype and idiotype representation in antibody responses have usually been treated as exceptional; however, studies partially summarized in Table VI demonstrate that epitope-specific regulation is demonstrable with diverse antigens administered under a wide variety of immunization conditions to many different mouse strains (from Herzenberg & Tokuhisa 1982). Furthermore, a retrospective view of the immunoregulatory literature indicates that the epitope-specific system plays a key role (albeit unrecognized as such) in regulating most of the commonly studied T-dependent antibody responses (see Herzenberg et al. 1982a). Thus we suggest that, in general, the characteristics of *in situ* antibody responses tend to be influenced more strongly by the epitope-specific regulation of memory B cell expression than by mechanisms that control memory B cell development (see summary in Table VII and discussion in Herzenberg et al. 1982a).

Some exceptions to the "rule"

Most of our studies of epitope-specific regulatory interactions have been

TABLE VII

*Requirements for optimal IgG antibody responses to epitopes on T-dependent antigens**

I. Memory B Cell Development:
- * Antigen reactive precursors (virgin B cells)
- * Carrier-primed T cells or presentation of antigens in a form that stimulates the development of carrier-primed T cells, e.g., on alum or in complete Freunds adjuvant (CFA)
- * Support for the IgD+ to IgD− memory shift

II. Memory B Cell Expression:
- * Helper T cells specific for the carrier on which the epitope is presented (or presentation of the antigen in a form that stimulates CTh development, e.g., on alum or in CFA)
- * Active prevention of the induction of epitope-specific suppression for each isotype/allotype response to a given epitope (e.g., priming with epitopes on newly-introduced carrier molecules usually prevents initial and subsequent suppression induction for responses to those epitopes)

little IgD, is present in only spleen and peripheral blood (missing in lymph nodes), appears first in ontogeny, and is highly represented in NZB mice.

This population (III) carries the "E2" marker, has less surface Ia (I-A) and mainly consists of cells that have small but clearly measurable amounts of the Lyt-1 antigen believed originally to be restricted to T cells (see Figure 14).

Further characterization of the Lyt-1 bearing B cells (Ly1 B) present in

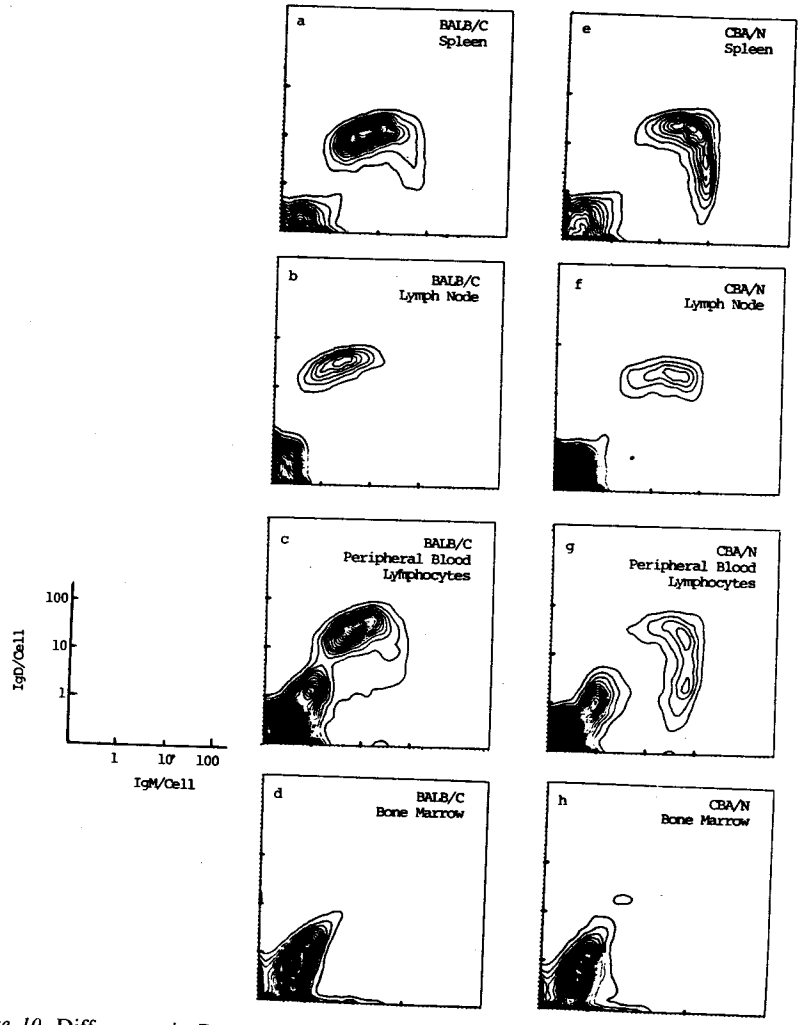

Figure 10. Differences in B cell subpopulation frequency in lymphoid organs from normal (BALB/c) and X-linked immunodeficient (CBA/N) mice. Population III is missing in both BALB/C (a–d) and CBA/N lymph nodes (e–h); population I is missing in all CBA/N organs.

subpopulation III demonstrates 1) that Ly1 B from NZB (but not from other) mice secrete relatively large amounts of IgM when cultured under standard conditions in the absence of exogenously-added antigens; 2) that these cells account for all of the IgM secreted by unseparated NZB spleen cells (or splenic B cells) cultured under conditions; and 3) that the NZB Ly1 B subpopulation does not contain "typical" IgM antibody producing cells (PFC) such as those generated following immunization with sheep erythrocytes (Hayakawa, Hardy and Herzenberg, manuscript in preparation).

The characteristic surface Ig levels on these subpopulations indicates that the quantitative expression of IgD and IgM on B cells is quite carefully regulated and thus suggests that each subpopulation has evolved mechanisms that permit it to express the amounts of IgD and IgM that suit its (currently unknown) function. This unsuspected orderliness raises a variety of new questions concerning the roles of IgM and IgD receptors and the internal regulatory mechanisms that selectively control receptor expression (e.g., does differential

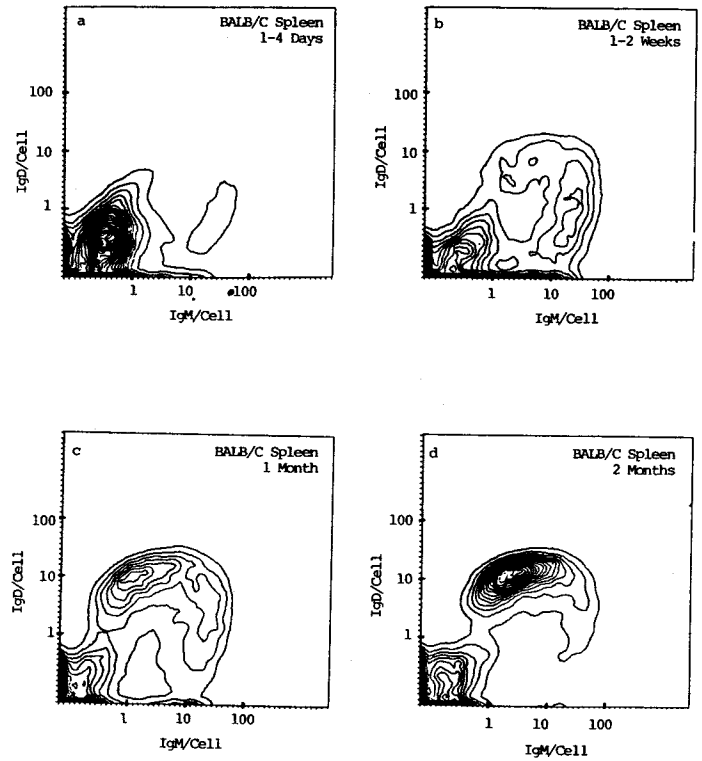

Figure 11. Population I appears late in spleen ontogeny. BALB/c spleen: (a) 1–4 days; (b) 1–2 weeks; (c) 1 month; (d) 2 months or older.

conducted with hapten-carrier conjugates such as DNP-KLH or TNP-TGAL that induce substantial IgG1, IgG2a, IgG2b and IgG3 anti-hapten antibody responses when used as priming antigens. The suppression induced by carrier/hapten-carrier immunization with any of these conjugates is effective in suppressing responses to the inducing hapten on any of the other conjugates and the isotype hierarchy described above appears to be maintained in all cases. Recently, however, we have been exploring the responses obtained with DNP

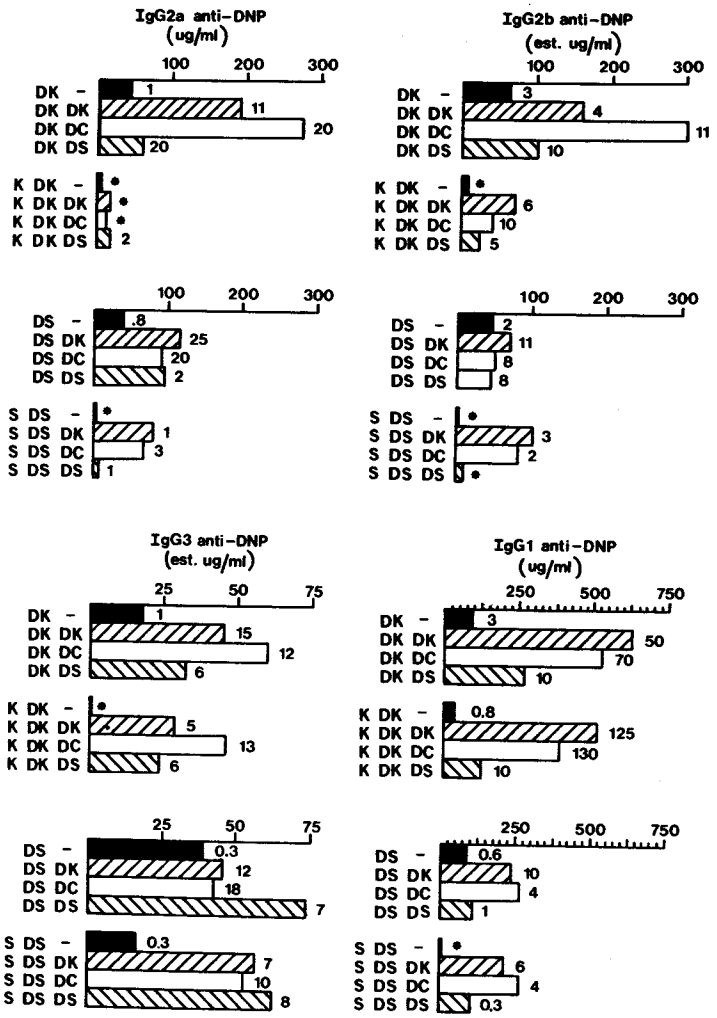

Figure 7a and 7b. Carriers that stimulate atypical isotype representation in anti-DNP responses (contrast with KLH or CGG) also stimulate atypical epitope-specific suppression.

conjugates that induce strikingly different initial isotype response patterns when used as priming antigens (see Figure 7). The results obtained with these conjugates (which may induce a substantial T-independent contribution to IgG responses) have added even more complexity to the regulatory picture.

In essence, carrier/hapten-carrier immunization with such conjugates induces specific and persistent suppression for anti-DNP responses when the DNP hapten is presented subsequently on the priming carrier; however, the suppression induced is relatively ineffective for preventing IgG anti-DNP responses when the suppressed animals are immunized subsequently with DNP-KLH. Furthermore, IgG3 rather than IgG1 responses tend to be the least suppressible when animals are immunized with these hapten-carrier conjugates (Hayakawa & Herzenberg, unpublished observations).

For example, priming BALB/C mice with high doses of DNP-coupled sheep erythrocytes (DNP-SRBC) induces substantial IgG3 anti-DNP responses, small but detectable IgG2a responses and virtually no IgG1 anti-DNP antibody production (see Figures 7a & 7b). Furthermore, SRBC/DNP-SRBC immunization induces specific suppression for IgG anti-DNP responses, i.e., anti-SRBC sponses proceed normally (data not shown) while anti-DNP antibody production remains minimal even when the animals are re-challenged with DNP-SRBC. Nevertheless, immunization with DNP-KLH at this point "breaks" or bypasses the suppression for IgG3 responses and stimulates the appearance of modest IgG2a and IgG1 anti-DNP responses as well.

We must admit that we have no explanation at present either as to how priming with DNP on these kinds of carriers induces such different isotype response patterns or how the suppression induced by carrier/hapten-carrier immunization with DNP on these carriers can be specific for anti-DNP responses when DNP is presented again on the priming carrier (anti-carrier responses are not affected) and yet minimally suppress anti-DNP responses to DNP on other carriers.

At the moment, we are exploring two approaches: examination of the memory B cell populations generated by these antigens to determine whether the response defect can be traced to influences on memory development rather than expression (experiments testing the induction of anti-DNP memory with DNP-SRBC administered under our immunization conditions are currently in progress); and, comparison of the anti-DNP idiotypes produced in the various isotype response to the antigens to determine whether non-overlapping repetoires are being accessed and independently regulated.

We therefore report these incomplete studies more for their caution than for their contribution. That is, data collected thus far show that although the epitope-specific system regulates reponses to a wide variety of antigens under broadly different immunization conditions, its properties (as formulated from responses to typical hapten-carrier conjugates) cannot explain all of the

peculiarities of isotype regulation in varied antibody responses stimulated by DNP presented on different kinds of carriers.

QUANTITATIVE REGULATION OF SURFACE ISOTYPE EXPRESSION

The amount of IgM present on B cells in normal lymphocyte populations spans roughly a 100-fold range (measured as the amount of fluorochrome-coupled anti-IgM/cell using a Fluorescence Activated Cell Sorter (FACS) fitted with logarithmic amplifiers). Similarly, the amount of IgD/cell in such populations spans about a 20-fold range. In both cases, the frequency distribution (FACS histogram) for cells stained with the relevant fluorochrome-coupled anti-Ig antibody has some irregular structure but is basically unimodal and appears to represent a continuum (see Figure 8). Nevertheless, our recent studies demonstrate clearly that these broad distributions are actually a composite of the distributions obtainable from at least three distinct B cell subpopulations identifiable by differences in the correlated expression of IgD and IgM on individual cells (see Figure 9) (Hardy et al. 1982a, b).

We defined these subpopulations initially using multiparameter FACS analyses with monoclonal antibodies to IgM and IgD; however, we soon found that they can be recognized by correlations with a variety of other characteristics (organ localization, genetically-controlled absences or increases, ontological differences, qualitative or quantitative differences in the expression of other surface markers) (see Figures 9–14).

For example, the major B cell subpopulation in adult spleen, lymph nodes and peripheral blood (population I) has relatively little surface IgM and intermediate to high levels of IgD. It is missing in CBA/N mice and uniquely lacks a B cell surface marker identified by a rat monoclonal antibody ("E2") (see fig. 12). The smallest subpopulation, in contrast, has abundant surface IgM and relatively

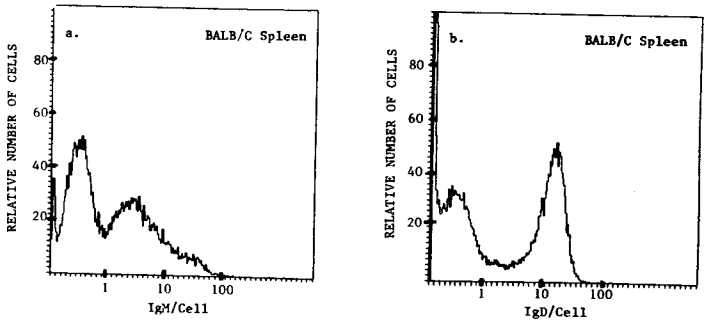

Figure 8. IgM and IgD expression on B cells. Single parameter FACS histograms for BALB/C spleen obtained with reagents used for multiparameter analyses shown in Figures 9–14.

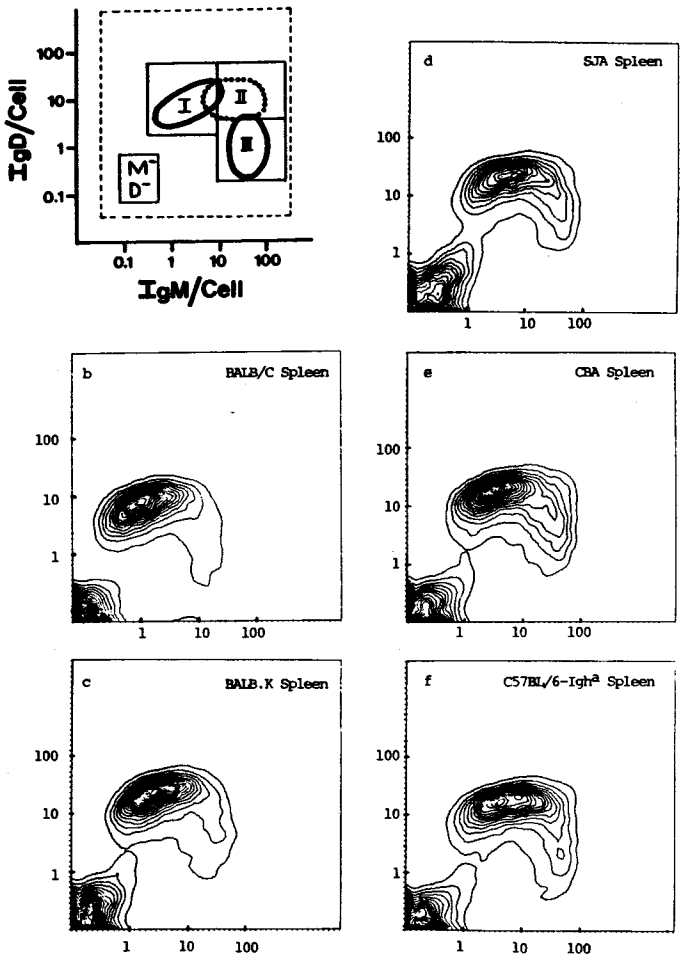

Figure 9. B cell subpopulations defined by IgM-IgD immunofluorescence staining and multiparameter FACS analysis. (a) schematic diagram; (b–f) FACS frequency "contour plots" showing subpopulation distributions in various mouse strains. Population II is less frequent in some strains: compare BALB/c and BALB.k (b,c) with SJA, CBA and C57BL/6 (d–f).

For these studies, we utilized a dual laser FACS to analyze correlations in the levels of two cell surface determinants revealed by the amounts of green and red fluorescence associated with individual cells after staining in suspension with a given pair of the following monoclonal antibody reagents (one "green" the other "red"): anti IgM #331-12 (Kincade et al. 1981); anti IgD 10-4.2, anti Ia 11-5 (Oi et al. 1978); anti ThB 49-h4 (Eckhardt and Herzenberg, 1980); "E2" 30-e2, anti Lyt-1 53-7.3 and anti Thy-1.2 30-h12 (Ledbetter and Herzenberg, 1979).

Our FACS instrument has two argon ion lasers: one is used both to generate a light scatter signal (size measurement) and to excite fluorescein (coupled directly to the "green" monoclonal antibody reagent); the second is used, in tandem with a dye laser, to excite a relatively new fluorescent dye, Texas Red (Molecular Probes, Plano, Texas) which is coupled to avidin and used as a second-step to reveal the biotin-labeled "red" monoclonal antibody reagent. Data presented in "contour plots" (in this and succeeding figures) show the amount of green fluorescence on individual cells along the X-axis and the amount of red fluorescence along the Y-axis, both in arbitrary units.