

FINE IDIOTYPE ANALYSIS OF B CELL PRECURSORS IN
THE T-DEPENDENT AND T-INDEPENDENT RESPONSES TO
 $\alpha 1 \rightarrow 3$ DEXTRAN IN BALB/c MICE*

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The humoral immune response of BALB/c mice to $\alpha 1 \rightarrow 3$ dextran (DEX)¹ has been well characterized with respect to isotype and idiotype expression. The serum antibody induced in BALB/c mice by DEX consists almost entirely of the IgM and IgG₃ heavy chain and lambda light chain isotypes (1, 2). The majority of serum antibody that binds DEX possesses a cross-reactive idiotype (IdX) present on both the J558 and M104E myeloma proteins and individual idiotypes (IdI) present on either J558 or M104E (3-5). The serum antibody from DEX-immune BALB/c mice had been previously shown to be homogeneous when examined with heterologous antiidiotype antibodies and by isoelectric focusing (4, 6). However, more recent amino acid sequence analysis of anti-DEX hybridoma antibodies has shown that while the majority of these antibodies are closely related, there is a considerable amount of heterogeneity mainly involving differences in only a few V_H region amino acid residues (7-9). Several mechanisms have been proposed for the generation of this limited amount of sequence diversity including imprecise joining of V-D-J gene segments, use of a large number of different D region genes and somatic mutation of variable region genes (9-12). It has also been suggested that somatic mutation of V segments is associated with isotype switching during B cell differentiation. This proposal is based on the observed increase in V_H sequence heterogeneity of IgG vs. IgM hybridomas that bind phosphorylcholine (13, 14).

We have used a panel of monoclonal antiidiotype antibodies (MAIDs) with distinct specificities to ask the following questions in relation to the above problems: (a) Do thymus-independent (TI) and thymus-dependent (TD) B cell precursors produce antibodies with differing idiotope profiles? (b) What effects do T cells have on the generation of clones secreting multiple immunoglobulin

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; DEX, dextran B1355S; DNP, dinitrophenyl; ELISA, enzyme-linked immunosorbant assay; Hy, Limulus hemocyanin; IdI, individual idiotype; IdX, cross-reactive idiotype; MAID, monoclonal antiidiotype antibody; TD, thymus dependent; TI, thymus independent.

isotypes? and (c) Within a single clone secreting more than one isotype, do the IgM and non-IgM molecules produced by differentiation of single clonal precursor express different idiotope profiles? By using this approach we planned to determine whether T cells play a role in the generation of idiotype diversity and to examine the time frame within which V region diversity may result from genetic events associated with immunoglobulin isotype switching.

Materials and Methods

Animals. Adult BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME and were used at ages 8–20 wk.

Antigens. Dextran B1355S (DEX), 35% α 1 \rightarrow 3 linkages, derived from *Leuconostoc mesenteroides* was a gift from Dr. Slodki (U.S.D.A. Agricultural Research Service). Dextran-Limulus hemocyanin (DEX-Hy) and dextran-bovine serum albumin (DEX-BSA) were gifts from R. Ward (University of Chicago) (15). Dinitrophenyl-ficoll (DNP-ficoll) was a gift from J. Quintans (University of Chicago). Limulus hemocyanin (Hy) was obtained from Sigma Chemical Co., St. Louis, MO.

Monoclonal Antiidiotype Antibodies. The construction of monoclonal antiidiotype antibodies has been described previously (16). Briefly, they were prepared by immunizing A/J, SJL/J, or BALB/c mice with purified J558 or M104E proteins. The lymph nodes were then fused with the nonsecreting myeloma Ag8.653 (16) and screened according to the protocol previously described (17). Antibodies reactive with J558 or M104E were then tested against a panel of purified myeloma proteins of each mouse isotype. The antibodies that appeared to react with idiotypic determinants on J558 or M104E were subjected to further analysis in a solid phase inhibition assay as described previously (17). Hybridoma lines secreting the monoclonal antiidiotype antibodies were cultured in RPMI 1640 with 20% fetal calf serum (Grand Island Biological Co., Grand Island, NY) and then purified by affinity chromatography appropriate for the particular isotype. Antibodies that were of the IgG_{2a} class were purified by elution from protein-A Sepharose 4B columns (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ). Antibodies of IgG₁ or IgM classes were purified by absorption to and elution from purified goat anti-mouse immunoglobulin coupled to Sepharose 4B (18). All antibodies were dialyzed against borate-buffered saline and stored in the same buffer (containing 0.1% sodium azide) at 4°C.

Splenic Focus Assay. The splenic focus assay was performed with minor modifications to that previously described by Klinman (19). Spleens from normal 8–10-wk old BALB/c mice were teased apart, washed, and passed over a glass-wool column. This single spleen cell suspension was transferred intravenously into lethally irradiated (1,400 rad) normal or Hy-primed recipients. 18–24 h later the recipient spleens were removed and diced into 1-mm cubes. The spleen fragments were then cultured in complete Dulbecco's modified Eagle's medium (Grand Island Biological Co.) with 10% fetal calf serum and antigen, in 96-well tissue culture plates (Costar, Data Packaging, Cambridge, MA) at 37°C. The optimum concentration of each antigen was initially determined by titration in several independent assays. Dextran B1355S was present in culture at 10 ng/ml while DEX-Hy was present in culture at 1 μ g/ml both from day 0 to day 4. TNP-Ficoll was injected intravenously into the recipient mice 1 h before dicing of the irradiated recipient spleens. Supernatants were harvested from each fragment culture every 3 to 4 d and assayed on days 15 or 19 by ELISA.

Assays for Anti-DEX Activity in Supernatants. Polystyrene (Costar) or polyvinyl microtitration plates (Dynatech Corp., Alexandria, VA) were coated with 3 μ g/ml DEX-BSA or with 10 μ g/ml of each MAID for at least 18 h at 4°C. Because of the small volume of supernatants (10 μ l) to be tested, it was necessary to centrifuge the 96-well microtitration plates after each addition to ensure symmetrical coating of the plastic wells. The plates were then blocked with 1% BSA in borate saline for 30 min at room temperature. 10 μ l of supernatant was added to each well and incubated at 4°C for at least 18 h. The appropriate alkaline phosphatase conjugated goat or monoclonal rat (anti- α) anti-mouse

isotype antibodies, prepared as previously described (16), were then added to each well and incubated for 3–4 h at 37°C. The wells were then washed and the (substrate) p-nitrophenyl phosphate, disodium (Sigma Chemical Co.) was added in diethanolamine buffer, pH 9.8. The optical density (405 nm) of each well was measured using an automated ELISA reader (Flow Laboratories, Rockville, MD). The limit of detection of anti-DEX antibody in this assay was about 10 ng/ml.

Results

Specificity of Monoclonal Antiidiotype Antibodies. A summary of the specificities of syngeneic (BALB/cJ) and allogeneic (A/J and SJL/J) MAIDs produced against J558 and M104E that were used in these experiments is shown in Table I. Each MAID has been shown previously to be hapten (nigerotriose) and DEX inhibitable (reference 5, and unpublished results). The DEX cross-reactive idiotope (IdX) is defined by CD3-2 (γ_1, λ), which recognizes determinants on both J558 and M104, and the majority of DEX-binding hybridomas produced in this laboratory and in the laboratory of Dr. B. Clevinger (Washington University). SJL18-1 (μ, κ) was produced against M104E and discriminates between M104E and J558 in ELISA inhibition assays. It also does not recognize any other myelomas or hybridomas contained within the J558 family (5). The remaining MAIDs EB3-7, B6-10, EB3-16, TD6-4, LA4-8, RD3-2, and JB2-2 were all prepared by immunization with J558, and do not bind M104E or hybridomas possessing the SJL18-1 idiotope. Each of these MAIDs was found to be distinct by their isoelectric focusing patterns and binding specificities (references 4 and 20, and unpublished results). Each MAID defines an idiotope present on J558 and recognizes subgroups within the EB3-7 positive family of anti-DEX hybridomas. Some of these idiotopes are also expressed by anti-DEX antibodies outside of the EB3-7 Idi family. A further point to be noted in Table I is that the idiotopes defined by this panel of MAIDs do not preferentially associate with any particular immunoglobulin isotype.

Frequency of Antigen-responsive B Cell Precursors. Since the clonality of the splenic focus assay is critical to the interpretation of data in this study, the number of donor cells injected into recipients was adjusted to give a plating

TABLE I
Specificity of Monoclonal Antiidiotype Antibodies to Hybridoma or Myeloma Proteins

MAID	J558 α, λ	3-19 γ_1, λ	8-21 γ_{2b}, λ	1-8 α, λ	M104 μ, λ	B14-1 γ_s, λ
CD3-2	+	+	+	+	+	+
EB3-7	+	+	+	+	–	–
LA4-8	+	+	+	+	–	–
TD6-4	+	+	+	–	–	–
EB3-16	+	+	+	–	–	–
RD3-2	+	+	–	–	–	–
JB2-2	+	+	–	–	–	–
B6-10	+	–	–	–	–	–
SJL18-1	–	–	–	–	+	+

* The specificities of monoclonal antiidiotype antibodies were determined in a solid phase ELISA as previously described (5).

efficiency of $\sim 10\%$ in all assays. At this plating efficiency only 0.53% of the wells would be expected by chance (Poisson distribution) to contain two or more DEX-positive B cell precursors. As a further test for clonality in this assay, B cell precursor frequencies for two independent antigens, DEX and DNP were analyzed within the same splenic fragments. The frequencies of DEX-positive and DNP-positive precursors in splenic focus assays stimulated simultaneously with both dextran B1355S and DNP-Ficoll are described in Table II. It can be seen that the frequency of DNP-positive foci is $\sim 30\%$ higher than the frequency of DEX-positive precursors. The observed frequency of foci secreting both anti-DNP and anti-DEX antibody was 1.52% of the total fragments, which is only slightly higher than the frequency of 1.16% expected from a chance association of independent DNP- and DEX-specific precursors. These results indicate that the homing of DEX-specific and DNP-specific B cell precursors to the spleen occurs randomly and that there do not appear to be certain sites within the spleen that are more conducive to individual precursor homing and maturation.

The frequencies of precursors responding to various TI and TD antigens (including DEX) have been previously described (15, 21). Assuming that B cells comprise $\sim 40\%$ of the adult spleen cell population and assuming a 4% homing efficiency (15, 22), the frequency of precursors responding to the TI antigen B1355S is ~ 1 in 1.28×10^5 and the frequency of precursors responding to the TD antigen DEX-Hy is ~ 1 in 7.04×10^4 .

Isotype Expression by T-independent and T-dependent B Cell Precursors. The in vivo serum antibody response of BALB/c mice to TI-2 antigens such as dextran B1355S has been reported to consist of antibody primarily of the IgM and IgG₃ isotypes (2). The response of carrier-primed mice to a TD antigen such as DEX-Hy has been shown to result in production of a larger proportion of antibody of the other IgG subclasses (23). Previous studies on isotype expression by clonal progeny of B cell precursors responding to TD antigens in vitro showed a high percentage of foci secreting more than one isotype, while the response to TI antigens consists of fewer multiple isotype secretors (21, 24, 25). Table III compares the isotype distribution of anti-DEX antibodies secreted by the progeny of B cell precursors stimulated by B1355S or by DEX-Hy. The most frequently

TABLE II
Frequency of Precursors Secreting Anti-DEX and Anti-DNP Antibody*

Assay no.	Foci examined	DEX ⁺ only	DNP ⁺ only	DEX ⁺ DNP ⁺	Expected doubles
1	288	32 [‡]	21	2	2.3 [‡]
2	192	30	27	6	4.2
3	192	18	46	4	4.3
4	288	22	35	4	2.7
5	288	15	26	3	1.4
Totals	1,248	117	155	19	14.5

* Splenic focus cultures were simultaneously stimulated with dextran B1355S and DNP-Ficoll and fragments secreting both anti-DEX and anti-DNP antibody were scored.

[‡] The number of fragments in each culture secreting antibody binding the appropriate antigen.

[§] The expected number of foci secreting both anti-DEX and anti-DNP antibody.

[‡] The differences between the values for expected and obtained values are all nonsignificant and *p* values are not shown.

TABLE III
Analysis of the Isotypes Expressed by Splenic Foci Stimulated by Dextran B1355S or DEX-Hy

Isotypes expressed	Antigen*	
	Dextran B1355S	DEX-Hy
IgM	89.7 (79.8) [‡]	42.9 (9.1)
IgG ₁	0	3.8 (1.3)
IgG _{2a}	0	0
IgG _{2b}	1.8 (1.3)	16.9 (2.6)
IgG ₃	2.7 (2.2)	0
IgA	13.5 (4.5)	81.8 (46.8)
2 isotypes	9.9	32.5
3 isotypes	0	5.2
4 isotypes	0	1.3

* The TI-2 antigen dextran B1355S or the TD antigen DEX-Hy were used to stimulate precursors in the splenic focus assay.

[‡] The percentage of DEX-positive foci expressing the appropriate isotype alone or in association with any other isotype. The number in parentheses indicates the percentage of foci expressing only the indicated isotype.

expressed isotype in response to B1355S was IgM, which was secreted by 90% of all DEX-positive foci. IgA was the next most frequently expressed (13.5%), followed by IgG₃ and IgG_{2b} at 2.7% and 1.8%, respectively. No IgG₁- or IgG_{2a}-producing clones were detected in the TI assays. Two-thirds of the IgA-positive foci in the TI assay also expressed IgM, suggesting that most of the IgA-positive foci may have originated from IgM precursors. B cell precursors stimulated by the TD antigen DEX-Hy showed a marked decrease in the number of foci secreting IgM anti-DEX antibody; only 42.9% were IgM positive, while the frequency of IgA anti-DEX precursors increased fivefold to ~81.8%. There was also an increase in the number of foci secreting IgG_{2b} and IgG₁, at 16.9% and 3.8% respectively. The frequency of IgG₃-positive anti-DEX foci showed little change when TI and TD assays were compared.

In addition to increased expression of most of the non-IgM isotypes in the TD assays, the frequency of foci secreting more than one isotype also increased, even though the overall frequencies of DEX-positive precursors were similar for both the TI and TD assays. The frequency of foci secreting two isotypes in the TI assay was ~9.9% of the total DEX-positive foci, and none secreted more than two isotypes. The frequency of foci secreting two isotypes in the TD assay was 32.5% of total DEX-positive foci, and 6.5% of the foci secreted more than two isotypes. In both the TI and TD assays the most frequently associated isotypes were IgM and IgA (data not shown). When the observed frequency of clones secreting multiple isotypes is compared with the predicted frequency of clones that would contain multiple isotypes resulting from two independent precursors it is apparent that most of the multiple isotype-secreting foci are progeny of one precursor and the result of class switching events (Table IV).

Idiotypic Expression of Antibody Secreted by B Cell Precursors Responding to Dextran B1355S and DEX-Hy. Although monoclonal antiidiotypic antibodies exhibit exquisite specificity, they cannot be used to define clonal markers on B cells since

TABLE IV
*Frequency of Multiple Isotype Secreting Foci Responding to the TD
 Antigen DEX-Hy*

Isotypes	Observed	Predicted
IgM-IgA	29.4*	3.11 [†]
IgM-IgG _{2b}	2.2	0.65
IgG _{2b} -IgA	3.7	1.23
IgM-IgG _{2b} -IgA	2.9	0.047
IgM-IgG ₁ -IgG _{2b} -IgA	1.5	0.0001

* The percentage of DEX-positive splenic foci secreting multiple isotypes in the TD assays.

[†] The predicted percentage of DEX-positive splenic foci secreting multiple isotypes.

TABLE V
*Analysis of the Idiotoxes Expressed by Splenic Foci Stimulated by
 Dextran B1355S or DEX-Hy**

Idiotope	Dextran B1355S	DEX-HY
EB3-7	74.1 [‡]	71.9
B6-10	2.3	4.7
EB3-16	6.8	14.0
TD6-4	17.3	29.0
LA4-8	59.0	55.1
RD3-2	4.2	12.7
JB2-2	4.8	5.1
SJL18-1	26.5	38.0
CD3-2	83.6	84.4

* The TI-2 antigen dextran B1355S or the TD antigen DEX-Hy were used to stimulate precursors in the splenic focus assay.

[‡] The percentage of DEX-positive splenic foci expressing the MAID-defined idiotope.

it has been shown previously in our laboratory and in others that most individual MAIDs bind a number of hybridoma and myeloma proteins that possess small but distinct differences in amino acid sequences (reference 20, and J. Kearney, unpublished results). However, by using a large panel of distinct MAIDs, an idiotope profile can be obtained for different clonally derived DEX binding antibodies from splenic foci. We have used our panel of MAIDs specific for DEX-binding proteins (Table I) to analyze, in fine detail, the expression of idiotopes on antibodies secreted by splenic foci stimulated with DEX and DEX-Hy. Table V illustrates independent expression of the various MAID-defined idiotopes on DEX-binding foci-derived antibodies. It is apparent that when the idiotopes expressed in the TI assays are compared to those expressed in the TD assays, no significant differences are apparent in the selection of idiotopes. These results indicate that generation of and/or selection of diverse idiotopes in the DEX system is independent of the presence of carrier-primed T cells.

The majority (84%) of TI and TD foci secreted antibody expressing the CD3-2 idiotope (IdX). ~73% of the foci expressed the EB3-7 idiotope (J558 IdI). Next most frequently expressed is the LA4-8 idiotope at 57%. The LA4-8

idiotope is also associated with a portion of both the J558 and M104E IdI families. The SJL18-1 idiotope (M104E IdI) is found on ~32% of antibody secreted by foci. Although the EB3-7 and SJL18-1 idiotopes defined two separate nonoverlapping groups of myeloma and hybridoma proteins, 8.5% of DEX-positive foci expressed both idiotopes. This is due in part to the random association of two different IdI-bearing precursors, and the other possibility is that the two idiotope families can on rare occasions overlap. The majority of DEX-positive antibodies expressed either the EB3-7 or SJL18-1 idiotopes, along with the cross-reactive CD3-2 isotope in agreement with previous analysis using heterologous antiidiotype antibodies that J558 and M104E-like antibodies constitute most of the λ anti-DEX response in BALB/c mice (3-5). The minor idiotopes EB3-16 and B6-10 (expressed by J558) are detected on only about 10% and 3% of DEX-positive foci. The EB3-16 idiotope is always associated with the EB3-7 idiotope, and the B6-10 idiotope is always associated with both EB3-16 and E3-7 idiotopes. EB3-16 and B6-10 defined smaller subsets within the J558 idiotype family. The TD6-4, RD3-2, and JB2-2 idiotopes are expressed on about 23%, 8%, and 5% of antibodies, respectively, and are usually associated with the J558 idiotope defined by EB3-7.

When the 209 anti-DEX-producing monofocal supernatants (from a total of 2349 splenic fragments) were assayed against the full panel of 9 MAIDs, 35 different idiotope profiles were obtained. Most foci-derived antibodies therefore expressed several idiotopes simultaneously, resulting in an idiotope profile that can be considered equivalent to idiotype. There was no preferential association of any idiotope profile with any particular isotype.

Intraclonal Idiotope Profiles of Monofocal Antibodies Differing in Isotypes. The diverse fine specificities of the MAIDs used in this study provided a method to detect minor differences in idiotopes expressed by distinct antibodies generated within individual splenic foci. Accordingly, if somatic mutation occurred frequently during the antigen-stimulated maturation of precursors to plasma cells, then the panel of MAIDs used in this study would be expected to detect some of these changes, if they occurred at critical residues and resulted in changes in idiotypes. The straightforward approach we used was to examine foci secreting more than one isotype and determine whether there were any differences in idiotope expression by the different isotypes detected within the same focus. A total of 45 double and triple isotype-secreting foci were examined for idiotope-isotype associations. 23 of these exhibited identical idiotope patterns for all isotypes expressed (several foci expressed only one or two idiotopes). In 22 foci containing multiple isotypes, distinct idiotope profile differences were detected on the IgM and non-IgM isotypes. Table VI lists all examples of multiple isotype-secreting foci in which differences were detected. Most of these differences were probably not due to the presence of more than one DEX-specific precursor in the original fragment, because of the low plating efficiency used. The frequency of fragments that would statistically be expected to contain more than one DEX-responsive precursor at a 10% plating efficiency is 0.53%. The frequency of foci coexpressing IgM and IgA by chance is ~3.1% of DEX-positive foci, which is 10-fold lower than the actual frequency of IgM-IgA doubles obtained (29.4%), suggesting that multiple isotype-secreting foci are the result of isotype switching.

TABLE VI
*Intraclonal Idiotope Profiles of Monofocal Antibodies Differing in Isotypes**

Focus	Isotypes	Idiotopes							
		EB37	B6-10	EB3-16	TD6	LA4	RD3	SJL18 [‡]	CD-3
D484	IgM	-	-	-	-	-	-	-	-
	IgA	+	+	+	-	-	-	-	+
D4C5	IgG _{2b}	+	-	+	+	+	+	-	+
	IgA	+	+	+	+	+	+	-	+
D6E12	IgM	+	-	+	+	+	+	-	+
	IgG _{2b}	+	-	+	-	-	-	-	+
	IgA	+	-	+	+	+	+	-	+
D6A8	IgG _{2b}	-	-	-	-	-	-	-	+
	IgA	-	-	-	-	-	-	+	+
D7A2	IgM	-	-	-	-	-	-	-	+
	IgG _{2b}	-	-	-	-	-	-	-	-
	IgA	+	-	-	-	+	-	-	+
D7F9	IgG _{2b}	+	-	+	-	+	-	-	+
	IgA	+	-	-	-	-	-	-	+
D7D7	IgM	+	-	+	+	+	-	-	+
	IgG _{2b}	+	-	-	-	-	-	-	+
	IgA	+	-	-	-	-	-	-	+
D7A12	IgM	+	-	-	-	-	-	-	+
	IgG _{2b}	-	-	-	-	-	-	-	-
D7E5	IgM	+	-	-	+	+	-	-	+
	IgA	+	-	+	+	+	-	-	+
D7B11	IgG _{2b}	-	-	-	-	-	-	-	+
	IgA	+	-	-	-	-	-	-	+
T10E11	IgM	+	+	+	-	-	-	-	+
	IgA	+	-	+	-	-	-	-	+
I11E11	IgM	-	-	-	-	-	-	-	+
	IgA	-	-	-	-	-	-	+	-
I12C1	IgM	+	+	-	-	-	-	-	+
	IgA	+	-	+	-	-	-	-	+
D73D7	IgM	-	-	-	-	-	-	?	+
	IgA	+	-	-	-	-	-	+	+
I16F8	IgM	-	-	-	-	-	-	?	+
	IgA	-	+	+	-	-	-	+	+
I4D10	IgM	+	-	-	-	-	-	-	+
I15C6	IgA	+	-	-	-	-	-	-	-
I17H5									
I18DF12									
D6C7	IgM	+	-	-	-	+	-	-	+
D6B7	IgA	+	-	-	-	-	-	-	+
D6H7									

* Monofocal supernatants were added to 96-well microtiter plates coated with all 9 MAIDs, and then developed with each AP-conjugated anti-mouse isotype corresponding to the isotypes expressed by each individual focus.

[‡] ? indicates that IgM reactivity with SJL18-1 could not be determined since SJL18-1 is of the IgM isotype; - indicates that the IgM containing focus in the SJL18-1 column contained no lambda bearing anti-DEX antibody bearing the SJL18-1 idiotope.

The idiotope differences detected on the different isotypes originating from one precursor could possibly be due to a competitive inhibition of binding by one isotype over another. Such a possibility is very unlikely because of the high coating concentration used in the ELISA, 10 $\mu\text{g}/\text{ml}$. The low concentration of antibody in the monofocal supernatants (100–400 ng/ml) did not saturate the MAID-coated wells. The possibility of competitive inhibition was also tested by mixing monofocal supernatants of different isotypes and known idiotope profiles. No inhibition was observed in any of these cases.

A further way of strengthening the evidence for the clonal origin of multiple isotype secreting foci is to examine the expression of the infrequent idiotopes on each of the isotypes secreted in one fragment (Table V). The EB3-16 idiotope is ideal for this purpose since it is expressed on only ~6–14% of DEX-positive foci. The frequencies (Poisson distribution) at which this idiotope would be expected to occur on DEX-positive antibodies secreted by two independent precursors is 0.098% for focus D4C5, 0.023% for focus T10E11, and 0.0005% for EB3-16 to appear on all three different isotypes secreted by focus D6E12. The actual frequencies at which the EB3-16 idiotope appears on individual isotypes is therefore 10–5000-fold higher than that expected. These statistical analyses strongly support the origin of multiple isotype secreting foci from one precursor cell. However, within these same foci it can be seen that there are idiotope shifts on the different isotypes detected.

The most likely explanation for the idiotope differences between the different isotypes within many of these foci is that they result from somatic variations occurring after in vitro antigenic stimulation and may be related to isotype switching events.

Discussion

The restricted and well-defined nature of the idiotope response of BALB/c mice to $\alpha 1 \rightarrow 3$ dextran provides a model in which to study the regulation and generation of antibody diversity (1–5). Amino acid analysis of hybridomas binding DEX (and other antigens) have shown that considerable heterogeneity exists between closely related families of antibodies binding the same antigen (8, 9, 14). However, these observed differences were usually limited to only a small number of amino acid residues. Somatic mutation has been proposed to account for this slight drift from the germline sequences, which may be an important mechanism for the generation of antibody diversity (9, 11–13). By examining the sequences of a number of hybridomas with our panel of MAIDs, a correlation between idiotope and certain amino acid residues can be made. For example, it has been shown that certain D region sequences were essential for reactivity with certain IdI-specific MAIDs and a glycosylated asparagine is important for reactivity with the IdX-specific MAID CD3-2 (reference 20, and B. Clevinger, personal communication). The same data also suggests that some amino acid substitutions can also be silent and have little or no effect on id-anti-id recognition. Each of the nine MAIDs used in this study is distinct (*a*) by isoelectric focusing (5), (*b*) by their reactivity with a large panel of anti-DEX hybridomas, and (*c*) by their reactivity with a preliminary set of monofocal supernatants (unpublished results). Since the substitution of as few as one residue can be

detected by appropriate MAIDs in some cases, we reasoned that this large panel of MAIDs would increase the likelihood of detecting minor idiotope shifts on monofocal antibodies that would occur as the result of somatic mutation. The primary goals of this study were: (a) to determine the potential heterogeneity of B cell precursors capable of responding to DEX, (b) to examine heterogeneity associated with each isotype expressed in response to DEX, and (c) to determine whether idiotope differences occur within foci that have undergone an isotope switch and that secrete more than one isotype.

The majority (Table III) of DEX-positive splenic foci stimulated by the TI form of the antigen secreted antibody bearing only the IgM isotype. Only $\sim 4.5\%$ of the foci secreted antibody of any of the IgG isotypes and only 13.5% secreted IgA, most of which also contained IgM. In contrast, only 9.1% of DEX-positive foci stimulated by the TD form of the antigen expressed IgM alone. An increase in detectable IgG and IgA isotypes was also apparent. There was also a fourfold increase in foci secreting two or more isotypes. The difference in isotypes of antibodies secreted by foci responding to TI or TD forms of α 1 \rightarrow 3 dextran, implies a direct role for carrier-primed T cells in regulating the expression of the various isotypes in the splenic focus assay. The serum of DEX-Hy-immunized BALB/c mice (Hy primed 6 wk earlier) contains little or no detectable IgA. The striking increase in IgA-secreting foci after stimulation by the TD antigen DEX-Hy may then be the result of T cell-mediated isotype switching or the result of enhanced IgA-specific T helper cell activity in this *in vitro* system. As suggested by others, the IgA-bearing B cell precursors may, *in vivo*, migrate to mucosal sites and as a result low levels of IgA would be expected to be found in serum (26, 27). The distribution of the various isotypes in the DEX TI assay is considerably different from the results published in other antigen systems, such as the TNP system (28). In the TNP (TI assays) system most foci expressed multiple isotypes, and a much higher frequency of IgG-secreting foci were detected. However, nude mice were used as recipients, a different antigen was used, and a higher frequency of positive foci was obtained.

An idiotope profile can be prepared for antibodies in each monofocal supernatant based on the reactivity of each individual MAID. A total of 35 different idiotope profiles were expressed by a total of 223 DEX-positive monofocal supernatants. This is probably a conservative indication of V region diversity within this group of DEX-specific clones, since not every amino acid sequence difference in individual proteins will be detected by this panel. Variations that are silent and do not affect idiotopes will not be detected. There appeared to be very little difference between the idiotope profiles of IgM, IgG, and IgA antibodies, indicating that there was no preferential association between an idiotope profile and any particular isotype. This is in contrast to data derived from amino acid sequence analysis of anti-PC hybridomas, which showed a greater degree of heterogeneity among IgG hybridomas vs. IgM hybridomas (13, 14). Greater heterogeneity may exist in IgG compared with IgM antibodies in these foci, but are not detectable by the panel of MAIDs used in this study. Despite this drawback it is quite evident that the IgM foci-derived antibodies already express considerable V region diversity unrelated to class switching events. The idiotopes that were detected by the monofocal antibodies in this

study correlated well with the frequencies of idiotopes expressed by anti-DEX hybridomas and detectable in DEX-immune sera of BALB/c mice. We have previously reported that the B6-10 idiotope is not detectable in DEX-immune sera of BALB/c mice (5). The reason for nonexpression of the B6-10 idiotope in sera is probably due to the very low frequency of responsive B6-10-positive precursors observed in our splenic focus assays. The B6-10 idiotope may be the result of an infrequently expressed germline gene, or the result of somatic variation of a germline gene.

One of the major questions to be asked in this study was whether any differences in idiotope profiles exist between the multiple isotypes secreted by the progeny of one precursor in any given fragment. It has been suggested that somatic variation may accompany a class switch by the activation of a hypermutation mechanism or somatic variation may occur throughout the life of a B cell with the result that older cells accumulate more mutations; these cells would also be more likely to have undergone a class switch (11–14). Splenic foci secreting more than one isotype provide a convenient means for examining the above hypothesis. Table VI clearly shows that in ~50% of the cases examined that the multiple isotype-secreting foci express different idiotope profiles. This should be considered a conservative estimate since not all differences will be detected by the panel of MAIDs used in this study. It must be concluded that in order for these idiotope differences to be detectable, the idiotope shift must have occurred about the same time as the class switch. If not, then the antibodies examined would consist of heterogeneous populations of idiotypes associated with each isotype, which would not be detectable in our assay. If the rate of somatic variation is very high with variation occurring well before or after class switching, then our assay may only be detecting somatic mutations that occurred by chance at about the same time as a class switch and are maintained thereafter during further clonal growth. A high degree of idiotype heterogeneity was also detected among the IgM precursors. This suggests that somatic variation may not always be associated with class switching. It is also evident that somatic variation involving B cell precursors which results in these distinct antibodies can occur within a very short time frame of less than 15 d, which is the time period during which the precursor B cells are cultured.

In conclusion, the splenic focus assay in conjunction with the use of MAIDs provides a powerful tool with which to probe possible mechanisms for the generation of antibody diversity. When comparing TI and TD assays, it becomes apparent that carrier-primed T cells are important in inducing expression of isotypes downstream from C μ . The presence of carrier-primed T cells does not appear to affect the expression of any particular idiotope or idiotope profile and the time frame in which the splenic focus assay is run (11–20 d) provides enough time for somatic mutation and subsequent idiotope changes to occur following antigenic stimulation.

What role does the generation of somatic variants play within the immune system? Are there redundant or degenerative antibodies? Isolation of clonally derived cell line variants by cell fusion procedures and structural analysis of immunoglobulins that they produce would provide more insight into the signif-

ificance of and the mechanisms involved in generation of such variant immunoglobulins.

Summary

In this study BALB/c B cell precursors responsive to the T-independent (TI) type 2 (TI-2) antigen, dextran B1355S (DEX), and the T-dependent (TD) derivative, dextran-Limulus hemocyanin (DEX-Hy) were examined for isotype and idiotope expression using the splenic focus assay. The predominant isotype detected in the TI assay was IgM, while IgA was the predominant isotype expressed in the TD assay. There was also a fourfold increase in the number of foci secreting more than one isotype in the TD assay vs. the TI assay without an overall change in anti-DEX precursor frequency, suggesting that carrier-primed T cells enhance the expression of non-IgM isotypes possibly by increasing the frequency of isotype switching by individual B cell precursors.

A panel of distinct monoclonal antiidiotope antibodies (MAIDs) was then used to examine idiotope expression by antibodies secreted in splenic foci responding to DEX and DEX-Hy. This analysis revealed considerable diversity in the idiotope profiles expressed by all isotypes tested. There appeared to be no differences in idiotope diversity among the various isotypes. A similar diversity of idiotope profiles was obtained from both TI and TD splenic foci, indicating that a comparable degree of diversity was associated with the antibodies generated by TI and TD precursors. Idiotope analysis of IgM-IgA-secreting foci with a panel of monoclonal antiidiotope antibodies revealed slight idiotypic differences between the two isotypes secreted in the same focus in about half the cases. These results suggest that somatic variation occurs during the antigen-driven maturation of B cell precursors, within the 15-d time frame of the splenic focus assay, and may be associated with isotype switching.

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