SUBCLASS RESTRICTION OF MURINE ANTIBODIES

II. The IgG Plaque-forming Cell Response to Thymus-independent Type 1 and Type 2 Antigens in Normal Mice and Mice Expressing an X-linked Immunodeficiency*

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Thymus-independent (TI)[1] antigens have been divided into two categories, TI-1 and TI-2, on the basis of their ability to stimulate an immunodeficient strain of mice, CBA/N, to produce antibodies (1, 2). These mice express an abnormality in B-lymphocyte development (3) which permits response to the TI-1 antigens, trinitrophenyl (TNP)-*Brucella abortus* (BA) (2), TNP-lipopolysaccharide (LPS) (4), and TNP-Nocardia (3), and not allowing response to the TI-2 antigens, TNP-Ficoll (5), phosphocholine (PC) (6, 7), and dextran (8).

We have recently determined that many of the antigens which fail to stimulate antibodies in CBA/N mice elicit responses in normal mice largely restricted to IgM and the IgG3 isotypes (9). Furthermore, CBA/N mice were shown to express preferential deficiencies of IgM and IgG3 immunoglobulins in serum and in spleen cells secreting these isotypes (10). This suggested that the CBA/N strain of mice may lack a subpopulation of B lymphocytes containing most of the precursors of IgG3-secreting cells. If this hypothesis is correct, then TI-1 and TI-2 antigens should stimulate antibodies of different isotypes. The present study confirms this prediction and provides further support for the subclass-specific nature of the CBA/N immunodeficiency.

Materials and Methods

Animals, Plasmacytomas, and Somatic Cell Hybrids. Male and female BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. CBA/N originally obtained from the Division of Research Services, National Institutes of Health and now maintained at Washington University were used to construct the (CBA/N × BALB/c)F₁ and (BALB/c × CBA/N)F₁ reciprocal crosses. All mice used were 3–4 mo of age. The BALB/c plasmacytomas J606(γ3,K), MOPC21(γ1,K), LPC1(γ2a,K), MPC11 (γ2b,K), J558(α,λ), and MOPC 104E

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[1] *Abbreviations used in this paper:* BA, *Brucella abortus*; DNP, dinitrophenol; GA-vaccine, group A streptococcal vaccine; GAC, group A streptococcal carbohydrate; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; PC, phosphocholine; PC-vaccine, heat-killed *Streptococcus pneumoniae* strain R36A; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TD, thymus-dependent; TI, thymus-independent; TNP, trinitrophenyl.

(μ,λ) were obtained from Litton Bionetics, Inc., Kensington, Md. under National Cancer Institute contract NO1-CB-92142, and maintained by serial passage in BALB/c mice. Somatic cell hybrids secreting IgG3 anti-α(1 → 3) dextran, IgG1 anti dinitrophenol (DNP), and IgG2b anti-DNP were generously provided by Brian Clevinger and Mitchell Scott of Washington University. These hybrids were generated by the fusion of mouse immune spleen cells and the transferase-deficient variety of the MOPC-21(γ1,K) line, NS1/1-Ag4-1, which synthesizes but does not secrete K chains (11) or SP2/0-Ag14, which does not synthesize either immunoglobulin chain (12).

Antigen Preparation and Immunizations.　Trinitrophenyl-*N*-(aminoethyl)-carbamylmethyl-Ficoll (TNP$_{26}$-Ficoll) consisting of 26 TNP groups/molecule of Ficoll (mol wt 400,000: Pharmacia Fine Chemicals Inc., Uppsala, Sweden) was made according to the method of Inman (13). TNP-BA was prepared with BA obtained from the U.S. Department of Agriculture Animal and Plant Health Inspection Services, Ames, Iowa (14) and TNP$_{16}$-LPS (*Escherichia coli* 0111: B4 LPS, Difco Laboratories, Detroit, Mich.) was prepared as described previously (15). TNP-keyhole limpet hemocyanin (KLH) and PC-KLH were prepared as before (16, 17) and had 16 and 65 groups per 10^5 daltons of protein, respectively.

Mice in groups of five were immunized with 100 μg TNP-Ficoll, 10^9 TNP-BA, 100 μg TNP-LPS, 100 μg dextran B1355 (obtained from Dr. M. Slodki, U. S. Department of Agriculture, Peoria, Ill.), or 10^8 heat-killed *Streptococcus pneumoniae* strain R36A (PC-vaccine) (18), all in saline intraperitoneally; responses were measured 7 d later. The response to sheep erythrocytes (SRBC) was measured 5 d after the fifth weekly injection of 0.1 ml of 2% washed SRBC intraperitoneally. Mice were hyperimmunized with group A streptococcal vaccine (GA-vaccine) as described previously (19) and the responses were measured 7 d after the fourth injection. Responses to TNP-KLH and PC-KLH were induced by an intraperitoneal injection of 100 μg antigen in complete Freund's adjuvant, followed in 1 mo by the same dose of antigen in saline: responses were measured 5 d later.

Isotype-specific Plaque Assays.　Spleen cells secreting antibody of IgM, IgG1, IgG2 (a+b), and IgG3 subclasses were detected in antigen-specific plaque assay systems with the use of subclass-specific facilitating antisera. SRBC were coated with group A streptococcal carbohydrate (GAC) (19), TNP (20), PC (18), and α(1 → 3) dextran (19) by previously described methods. The preparation of rabbit anti-γ3 was described previously (10); rabbit anti-γ2 and anti-γ1 were purchased from Gateway Immunosera, Cahokia, Ill. and rendered subclass-specific by solid-phase immunoabsorption. Specificity and efficiencies of the facilitating antisera were verified using a panel of mouse plasmacytomas and/or hybridomas secreting antibody of the major mouse immunoglobulin isotypes (Results). The panel was tested for both antigen-specific plaque-forming cells (PFC) and for isotype-secreting cells (reverse plaque assay) using staphylococcal protein A-coupled SRBC (21). Facilitating antisera diluted approximately 1:2,000 were found to give near optimal plaquing efficiencies. To detect only IgG-secreting spleen cells in the antigen-specific plaque assays, anti-μ (anti-M104 absorbed with a J558-Sepharose column to remove anti-idiotype and anti-λ) was included in the agarose to suppress direct (IgM) PFC. The absorbed anti-μ at a 1:2,000 dilution suppressed M104 plasmacytoma cells at densities up to 10^5 cells per slide but showed no suppression of plasmacytoma cells secreting antibody of the other major mouse isotypes.

Results

Specificity of Facilitating Antisera in PFC Assays.　The availability of panels of monoclonal cell lines each secreting antibodies of a single class provides an excellent control system to document the specificity of reagents in the plaque assay. Table I summarizes the efficiency and specificity of the facilitating antisera used in this study. It can be seen that each antiserum is highly specific; both anti-γ1 and anti-γ3 have > 10^4-fold specificity for their respective isotypes; anti-γ2 cross-reacts slightly with one of two γ1-secreting lines and does not distinguish between γ2a and γ2b determinants.

Isotype Distribution of Antibodies to TI-1, TI-2, and Thumus-dependent (TD) Antigens. Conventionally, TI antigens are considered to stimulate predominantly IgM antibod-

TABLE I

*Specificity of Anti-Isotype Antisera**

Hybridoma and plasma-cytoma cell lines	Class	Antigen specificity	Plaque assay		Facilitating antisera		
			Antigen-specific	Isotype-specific	Anti-γ1	Anti-γ2	Anti-γ3
					PFC/10² cells (SE)		
H3-12-D	γ1	DNP	+		45 (± 2)	0.5 (± 0.08)	<0.001
MOPC-21	γ1	?		+	47 (± 6)	<0.001	<0.001
LPC 1	γ2a	?		+	<0.001	24 (± 3)	<0.001
H3-30-E	γ2b	DNP	+		<0.001	53 (± 7)	<0.001
MPC 11	γ2b	?		+	<0.001	24 (± 4)	<0.001
H26d2d4	γ3	α(1 → 3)-Dextran	+		<0.001	<0.001	30 (± 3)
J606	γ3	Levan		+	<0.001	<0.001	45 (± 5)
J558	α	α(1 → 3)-Dextran	+		<0.001	<0.001	<0.001
J558	α	α(1 → 3)-Dextran		+	<0.001	<0.001	<0.001
MOPC 104	μ	α(1 → 3)-Dextran	+		<0.001	<0.001	<0.001
MOPC 104	μ	α(1 → 3)-Dextran		+	<0.001	<0.001	<0.001

* Hybridoma or plasmacytoma cells grown in tissue culture were tested for plaque-forming ability against antigen-coated or protein A-coated erythrocytes after exposure to facilitating antisera specific for γ1, γ2, and γ3 determinants. Cells were tested at several densities up to 10^5 per slide at optimal concentrations of facilitating antisera.

ies, although exceptions are well known. Furthermore, our laboratory has reported the absence of facilitated PFC to PC-vaccine and GAC even though serum IgG antibodies were known to exist (18, 19). Thus, it was important to develop efficient methods for detecting IgG PFC to polysaccharides, as well as other determinants. This was accomplished in these studies by facilitating with subclass-specific antisera so that optimal conditions for detecting each subclass were ensured. It can be seen in Table II that IgM and IgG antibodies were elicited by all TI-1, TI-2, and TD antigens tested. Immunization protocols were chosen to given adequate numbers of IgG PFC for subclass determination, and not necessarily to give maximum PFC responses.

When the distributions of the IgG subclasses were determined, it was found that each class of antigen stimulates distinctive patterns of isotype preference (Fig. 1). TI-1 antigens stimulate IgG2 and IgG3 antibodies in similar amounts, whereas TI-2 antigens stimulate predominantly IgG3. TD antigens, on the other hand, elicit predominantly IgG1 with smaller amounts of the other isotypes. These results confirm our previous findings which compared purified serum antibodies to TI-2 and TD antigens (9).

Response of the CBA/N Mouse to TI-1 and TI-2 Antigens. Although it is clear that TI-1 and TI-2 antigens stimulate different patterns of response, both stimulate IgG3 antibodies. Because our previous studies had suggested that the immunodeficiency of the CBA/N strain of mice was reflected chiefly in the ability to produce IgG3 antibodies (10), it was of particular interest to examine the subclass distribution of the antibodies made by CBA/N mice to TI-1 antigens. Shown in Fig. 2 is a comparison of the responses of (CBA/N × BALB/c)F₁ defective male mice to (BALB/c × CBA/N)F₁ nondefective males to both TI-1 and TI-2 antigens. The results are clear. The defective animals have selective deficiencies in IgG3 antibody response to both classes of antigen. Thus, 90% or more of the IgG3 response to TI-1 antigens is absent in defective animals, whereas almost no change is seen in IgG2 or IgM components.

TABLE II

IgM and IgG PFC responses to TI-1, TI-2, and TD antigens

Immunogen	Immunization protocol*		n	Antibody response	
	1°	2°		Direct PFC/spleen	Indirect PFC/spleen
TNP-BA	10^9	—	5	9,500 ± 3,000	11,300 ± 1,400
TNP-LPS	100 µg	—	5	30,900 ± 8,700	54,300 ± 8,100
TNP-Ficoll	100 µg	—	5	20,100 ± 5,100	14,300 ± 2,800
α(1 → 3) Dextran	100 µg	—	5	9,300 ± 1,500	17,500 ± 1,500
PC-vaccine	10^8	—	5	11,900 ± 2,800	5,000 ± 1,600
GA-vaccine	Hyperimmunization		5	192,600 ± 62,500	41,300 ± 21,000
SRBC	Hyperimmunization		5	2,200 ± 300	17,200 ± 1,800
TNP-KLH	100 µg(CFA)	100 µg	5	3,500 ± 400	103,100 ± 15,600
PC-KLH	100 µg(CFA)	100 µg	5	48,100 ± 10,800	51,200 ± 12,100

* Groups of five BALB/c mice were immunized intraperitonally with the antigens listed. Consult Materials and Methods for details of immunization. PFC responses were measured 5–7 d after the last antigen administration. Shown are the geometric means and standard errors of the direct and indirect PFC: the indirect PFC values represent the sum of the individual IgG isotypes detected.
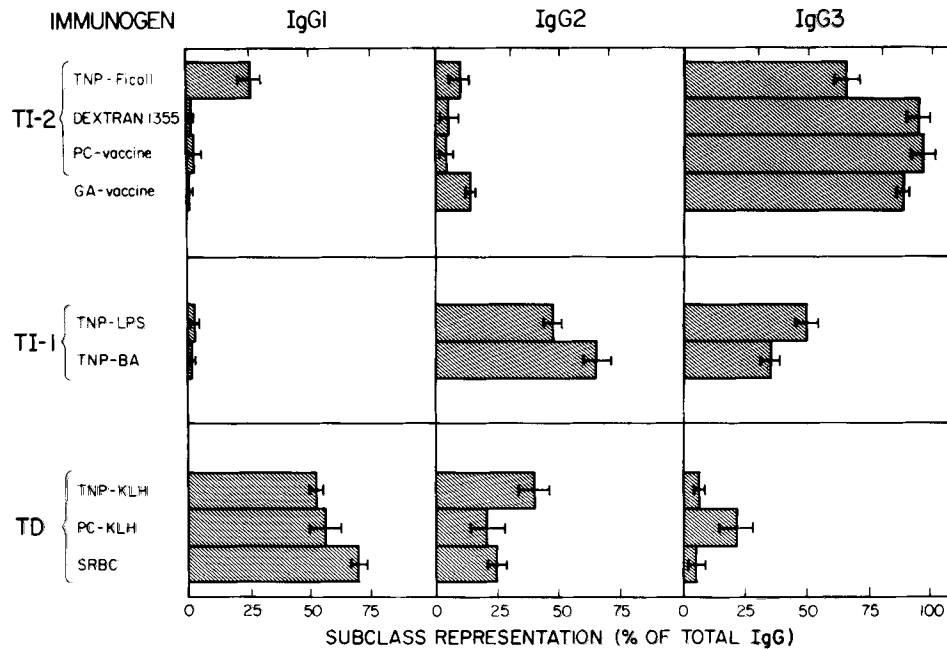

FIG. 1. Antibody isotype distribution in response to TI-2, TI-1, and TD immunogens. Animals in groups of five were immunized as described in Table II. Shown here are the geometric means and standard errors of the antigen-specific PFC facilitated with anti-γ1, -γ2, and -γ3 antisera, normalized to the total indirect PFC response.

Clearly, the response by defective animals to TI-1 antigens is not normal. However, the distinction between TI-1 and TI-2 antigens seems not to be limited to the IgG2 subclass because the IgM, as well as the IgG3, response to TNP-Ficoll is absent in defective mice, whereas the IgM responses to both TI-1 antigens are preserved.
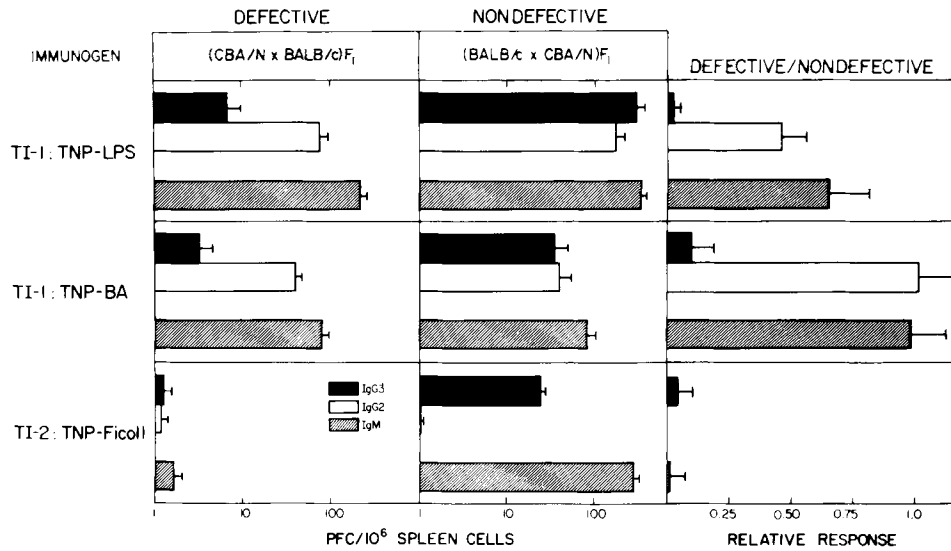
Fig. 2. The IgG PFC response to the TI-2 antigen TNP-Ficoll and to the TI-1 antigens TNP-BA and TNP-LPS in normal (BALB/c × CBA/N)F₁ males and (CBA/N × BALB/c)F₁ males expressing an X-linked immunodeficiency. Normal (BALB/c × CBA/N)F₁ males and immunodeficient (CBA/N × BALB/c)F₁ male mice in groups of five were immunized with TNP-LPS, TNP-BA, or TNP-Ficoll as described in Table II. 7 d later, IgM, IgG1, IgG2, and IgG3 anti-TNP PFC were measured in the spleen; shown are geometric means and standard errors.

Discussion

The TI-2 antigens TNP-Ficoll, dextran, PC-vaccine, and GA-vaccine generate IgG antibody responses predominantly, but not exclusively, of the IgG3 subclass. This fact may explain the difficulty encountered in the past in detecting indirect PFC to these antigens (18, 19, 22). It is likely that the usual polyspecific facilitating antisera have little activity against the rare subclass IgG3. However, even when subclass-specific facilitation was performed in the study of responses to these antigens, only the more common IgG subclasses were evaluated. Thus, only the minor components of the IgG response to DNP-Ficoll were noted by PFC assays in earlier studies (23, 24); however, a recent study by Venkataraman and Scott disclosed IgG3 PFC to fluorescein-Ficoll (25). Furthermore, significant cross-reactivity between IgG subclasses can occur in antisera to IgG and failure to remove cross-reactive antibodies could result in mistaken identification of subclass. Serologic analyses of antibodies to TI-2 antigens have generally not evaluated IgG3 components (26–28) until recently (9), so that the dominance of this isotype has not been appreciated.

The TI-1 antigens, TNP-LPS and TNP-BA, stimulate antibody responses which are different from those to TI-2 antigens. Both TI-1 antigens tested stimulate about equal proportions of IgG2 and IgG3 in addition to IgM. Similarly, responses to TD antigens are unlike those to TI-1 and TI-2 antigens in that IgG1 antibody is predominant. Thus, categorization of antigens on the basis of thymus dependence and on the ability to stimulate an immunodeficient strain of mouse seems to reflect different preferences of the antigens for IgG isotypes.

Furthermore, it is reasonably clear that different categories of antigens stimulate different subpopulations of B cells. Limiting-dilution analysis has shown that different

cell populations respond to TNP-Ficoll, TNP-LPS, and TNP-erythrocytes or TNP-protein (29), although some degree of cross-stimulation can be demonstrated (30). The different subpopulations become immunocompetent at different times of ontogeny (31, 32) and express size differences (33), as well as distinctive differentiation antigens (34, 35). Thus, it is well established that heterogeneity exists among B cells. Therefore, one interpretation of the results of this paper is that TI-1 antigens stimulate two separate subpopulations of B cells, one restricted to IgM and IgG2, the other restricted to IgM and IgG3; TI-2 antigens stimulate only the latter subpopulation. TD antigens stimulate chiefly a third subpopulation of B cells restricted to IgM and IgG1 production. Thus, the X-linked defect of the CBA/N mouse in the development of the IgG3 precursors results in a complete failure to respond to TI-2 antigens, a partial failure to respond to TI-1 antigens and little effect on the response to TD antigens.

Although heterogeneity among B cells is established, the interrelationships of these subpopulations to one another is not clear. One possibility is that there is a single pathway of differentiation with recognizable intermediate stages of B-cell development (36), each responsive to different types of antigens, and possibly each generating distinct subclasses of immunoglobulins. This model further predicts that some intrinsic property of an antigen, not related to the particular antigenic determinants binding to cell receptors, stimulates selectively a B cell at a particular stage of development to produce a given class of immunoglobulin. Changing the nature of the carrier from, for example, TNP-Ficoll to TNP-KLH may activate the same clones of cells, but at different stages of development. In other words, antigen and relevant accessory cells like T helper cells (37) select the antibody subclass produced by stimulating B cells at particular stages of differentiation. Furthermore, variable regions would most likely be found equally distributed among the various B-cell subpopulations.

The alternative model, which we favor, postulates separate lineages of B cells, each limited with respect to antigen responsiveness (38) and isotype expression. For example, the late-appearing B cell absent from CBA/N mice would be restricted to the production of IgG3 of the IgG subclasses as well as IgM and probably other classes (10). One may further postulate that if indeed C_H gene expression is developmentally segregated, then V_H expression may be segregated as well. Such a mechanism may restrict the expression of particular C_H genes and could explain the restriction of responses to various antigens to particular subclasses.

Thus, subclass restriction according to this model would reflect restriction imposed by V_H-C_H pairing. This we feel is the central issue distinguishing the models of B-cell development. Do antigens elicit antibodies of restricted subclass because of properties intrinsic to the antigen unrelated to its ability to bind to the B-cell receptor (such as mitogenicity, ability to stimulate T cell help, molecular dimensions, etc.) or because subclasses are clonally restricted? Although it is not possible to distinguish between these models at present, we can examine the existing relevant data for support of either model.

First, are there common physicochemical features which identify immunogens as TI-1, TI-2, or TD? At first glance, there does appear to be commonality: TI-1 antigens are polyclonal stimulators (2), TI-2 antigens are simple polysaccharides, and TD antigens are hapten-proteins and cell membrane antigens. However, there are important exceptions. For example, coupling GAC to protein does not alter the isotype of

the antibodies to GAC significantly (9). This is best demonstrated, however, in the rat where immunization with p-azobenzenearsonate-KLH and DNP-KLH stimulates anti-hapten responses of the IgG2a subclass, whereas PC-KLH, like PC-vaccine, stimulates only IgG2c,[2] the rat analogue of IgG3.[3]

Thus, on the basis of subclass preference, PC-KLH stimulates responses in the rat indistinguishable to PC-vaccine or many other polysaccharides and would require classification on this basis as a TI-2 antigen. Although this seems not to be the case in mice (Fig. 1), work to be presented elsewhere demonstrates that the PC-specific antibodies of different subclasses largely have different variable regions. Thus, although similarities exist for many antigens within an antigen class, the several exceptions suggest that no single feature of antigen structure regulates isotype preference.

This then raises the possibility that antigens select IgG subclasses on the basis of selective interaction with B-cell receptors which themselves are distributed nonrandomly. In other words, the reason anti-$\alpha(1 \rightarrow 3)$ dextran antibodies are predominantly IgM and IgG3 is because variable regions specific for these determinants are expressed only by the B-cell subpopulation restricted to these isotypes. What is the evidence with regard to randomness of V_H-C_H pairing? Although there is no doubt that single V_H regions can pair with $C\mu$, $C\alpha$, $C\delta$, and $C\gamma$, only a few studies have examined whether V_H regions can pair with multiple $C\gamma$ subclasses. Single clones of cells secreting anti-DNP antibody frequently produce both IgG1 and IgG2 subclasses in splenic focus culture (39). Furthermore, the bulk of anti-poly-(L-glutamic acid[60], L-alanine[30], L-tyrosine[10]) antibodies in mice are IgG1 (40) although minor levels of IgG2 and IgG3 antibodies have been detected (41). Antibodies of all three subclasses react with a heterologous anti-idiotypic antiserum known to have broad specificity (41). Similarly, Claflin and Cubberley have demonstrated that anti-PC antibodies raised to PC-KLH in mice are distributed among IgG1, IgG2, and IgG3 subclasses and all share idiotypic determinants with the PC-specific myeloma protein, T15 (42). How are these data compatible with the hypothesis of nonrandom V_H-C_H joining? First, one can argue that sharing of idiotypic determinants indicates similarity, but not necessarily identity of variable region structure. Numerous examples exist of immunoglobulins with different specificities or different variable region sequences sharing idiotypic determinants (43, 44). In fact, recent work from this laboratory demonstrates that differences do exist between idiotypes on anti-PC antibodies of different IgG subclasses. However, this argument does not pertain to data derived from splenic-focus assays where the antibodies are derived from a single precursor. Similarly, the successful isolation of variant myeloma lines in which the same variable regions are paired with both IgG2a and IgG2b argue for random pairing for at least these subclasses (45). In this latter case, however, structural analysis of the mutant proteins demonstrate atypical recombination of V_H and C_H so that the generality of the observations is uncertain (46).

It is obviously premature to decide on a particular model of B-cell differentiation. However, we suggest that the categorization of antigens into subclasses of TI and TD, although operationally useful, implies special properties of the antigen. It is equally

[2] Der-Balian, G. P., J. Slack, B. Clevinger, H. Bazin, J. M. Davie. Manuscript in preparation.
[3] Nahm, M., G. P. Der-Balian, H. Bazin, and J. M. Davie. Manuscript in preparation.

likely that B cells are the principal regulators of the pattern of antibody responses. Furthermore, because limited surveys of antigens (such as presented in this paper) suggest discrete patterns of response, it will not be surprising if many intermediate patterns will be detected as more antigens are tested.

Summary

Antigens have been classified previously into three categories, thymus-dependent (TD), thymus-independent type (TI) 1, and TI-2, based upon thymic dependence and ability to stimulate an immunodeficient strain of mouse, CBA/N. Here we demonstrate that the different antigen classes elicit IgG antibodies of different subclasses. TD antigens stimulate predominantly IgG1 antibodies, with smaller amounts of IgG2 and IgG3 being expressed. TI-1 antigens stimulate almost no IgG1 antibodies and equal amounts of IgG2 and IgG3. TI-2 antigens elicit predominantly IgG3 antibodies. Mice expressing the CBA/N phenotype are known to be nonresponsive to TI-2 antigens. This was confirmed in this study. In addition, we demonstrate that the IgG3 component of the response to TI-1 antigens is virtually absent in mice expressing the CBA/N phenotype, which supports our previous finding that the CBA/N defect may be restricted to a B-lymphocyte subpopulation containing most of the precursors of IgG3-secreting cells.

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