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SUBCLASS RESTRICTION OF MURINE ANTIBODIES

III. Antigens That Stimulate IgG_3 in Mice Stimulate IgG_{2c} in Rats*

By GEORGES P. DER BALIAN,‡ JOHN SLACK,§ BRIAN L. CLEVINGER,‖ HERVÉ BAZIN, AND JOSEPH M. DAVIE

From the Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110; and the Experimental Immunology Unit, University of Louvain, Brussels, Belgium

The evolutionary relationships between subclasses of IgG occurring in different species of animals are uncertain. One hypothesis, based on amino acid sequences, suggests that some IgG isotypes developed late, after speciation (1, 2), and that the similarities in functional specialization result from parallel mutation (3). On the other hand, serologic analysis of human subclasses demonstrated that one of the four subclasses, IgG_2, shared antigenic determinants with immunoglobulins of other primates and might be a more primitive subclass (4). Furthermore, Yount et al. (5) first demonstrated that polysaccharide but not protein antigens stimulated preferentially this subclass of IgG.

In previous papers in this series, we have demonstrated that similar restrictions in subclass occur in mice (6) and that antigens can be categorized according to which of the four IgG subclasses is elicited (7). IgG antibodies to protein, hapten-protein conjugates, or sheep erythrocytes are mainly IgG_1. On the other hand, IgG antibodies to carbohydrates or hapten-carbohydrate conjugates are largely IgG_3, a subclass found in normal mouse serum in very low amounts (8). Here we demonstrate an analogous subclass preference in rat antibody responses. By comparing subclass preference in rats and mice with a variety of antigens, it is possible to identify functional analogues among subclasses. In particular, antigens that stimulate IgG_3 in mice stimulate IgG_{2c} in rats. Furthermore, as we will show elsewhere, these subclasses share antigenic determinants.[1] Such functional and serological similarity implies a close evolutionary relationship between these IgG subclasses. Thus, it is likely that at least some IgG subclasses arose before rat-mouse speciation.

Materials and Methods

Animals. Young adult outbred albino rats of either sex (NLR strain; National Laboratory Animal Company, O'Fallon, Mo.) were used.

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[1] Nahm, M., G. P. Der Balian, D. Venturini, H. Bazin, and J. M. Davie. Antigenic similarities of rat and mouse IgG subclasses associated with anti-carbohydrate specificities. *Immunogenetics.* In press.

Antigens and Immunization. Keyhole limpet hemocyanin (KLH)[2] was purchased from Calbiochem-Behring Corp. American Hoechst Corp., (San Diego, Calif.); dinitrophenyl-L-lysine, bovine serum (BSA), and egg albumins (OVA) came from Sigma Chemical Co. (St. Louis, Mo.); and Ficoll (~400,000 daltons) came from Pharmacia (Uppsala, Sweden). Dextran B1355 was a generous gift from Dr. M. E. Slodki (U. S. Dept. of Agriculture, Peoria, Ill.).

Group A (GA-) and group C (GC-)streptococcal vaccines and *Streptococcus pneumoniae* strain R36A (PC-vaccine) were prepared as described previously (6, 9). Dinitrophenyl conjugates of carbohydrates (DNP_6-dextran B1355 and DNP_6-Ficoll) were prepared according to Inman (10) and conjugates with proteins (DNP_4-OVA and DNP_{13}-KLH) according to Little and Eisen (11). Trinitrophenyl *Brucella abortus* (TNP-BA) and trinitrophenyl lipopolysaccharide (TNP_{16}-LPS) were prepared as before (12, 13). Phosphocholine (PC_{65})-KLH was prepared by the method of Chesebro and Metzger (14). *p*-Azobenzenearsonate (ABA)-KLH was prepared by the method of Nisonoff (15).

Details of immunization are shown in Table I. The animals were bled 1 wk after the last immunization (for serum isotypic determination) or spleens were analyzed for plaque-forming cells (PFC) 5–7 d after the last immunization.

Preparation of Immunoadsorbent Beads and Antibody Purification. *N*-Hydroxysuccinimide esters of carboxylated Sepharose 4B beads (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were prepared according to the methods of Inman (10) and Cuatrecasas and Parikh (16). DNP beads were prepared by suspending 10-ml esterified beads in a neutral saturated solution of DNP-L-lysine in phosphate-buffered saline (PBS: 0.15M NaCl and 0.01 M potassium phosphate, pH 7.4) for 12 h at 4°C. ABA beads were prepared by adding 1 mmol of the diazonium salt of arsanilic acid to 10 ml of BSA-coupled beads in 50 ml of borate-buffered saline (BBS: 0.035 M

TABLE I

Summary of Immunization Protocols and Antibody Responses

Immunogen	Immunization protocol			*n*	PFC response§	
	Primary	Secondary*	Tertiary‡		Direct PFC/ spleen	Indirect PFC/ spleen
PC-vaccine	5×10^8 R36a i.p.	—	—	4	9,150 (1.4)	29,800 (1.3)
PC_{65}-KLH	200 μg (CFA) i.p.	200 μg		5	17,400 (1.5)	131,900 (3.3)
GA-vaccine‖	30 μg rhamnose i.p.	30 μg	—	3	63,400 (1.2)	16,800 (2.4)
GC-vaccine‖	30 μg rhamnose i.v.	30 μg	30 μg	6	Serum analysis only	
DNP_{26}-Ficoll	200 μg i.p.	—	—	5	12,500 (1.5)	29,400 (2.2)
DNP_6-Dextran	1,250 μg i.p.	—	—	4	133,500 (1.5)	265,600 (1.7)
DNP_4-OVA	200 μg (CFA) i.p.	200 μg	—	5	3,400 (1.5)	37,700 (1.5)
OVA	200 μg (CFA) i.p.	200 μg	—	2	<100 (1)	60,800 (1.3)
ABA-KLH	300 μg (CFA) i.p.	250 μg	—	5	Serum analysis only	
DNP_{13}-KLH	1,000 μg (CFA) i.p.	500 μg	—	3	Serum analysis only	
KLH	250 μg (CFA) i.p.	250 μg	—	8	Serum analysis only	
TNP-BA	5×10^9 organisms	—	—	3	27,200 (4.1)	176,100 (1.1)
TNP-LPS	200 μg i.p.	—	—	3	11,300 (2.2)	39,000 (1.5)

* Secondary immunization was 4 wk after the primary.

‡ Tertiary immunization was 5 wk after the secondary.

§ Spleens were analyzed for antigen-specific PFC 5–7 d after the last immunization. Indirect PFC are the sums of the individual IgG isotopes detected. The geometric means and standard errors are shown. Those responses analyzed by serum antibody alone were from serum pools taken 7 d after the last immunization.

‖ The dose of GA- and GC-vaccine injected was calculated on the basis of rhamnose, a cell-wall constituent.

[2] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; BBS, borate-buffered saline; BSA, bovine serum albumin; DNP, dinitrophenyl; GAC, group A carbohydrate; GA-vaccine, group A streptococcal vaccine; GC-vaccine, group C streptococcal vaccine; KLH, keyhole limpet hemocyanin; OVA, egg albumin; PBS, phosphate-buffered saline; PC_{65}, phosphocholine; PC-vaccine, *Streptococcus pneumoniae* strain R36A vaccine; PFC, plaque-forming cell(s); TI-1, thymus-independent type 1 antigen; TI-2, thymus-independent type 2 antigen; TNP-BA, trinitrophenyl-*Brucella abortus*; TNP16-LPS, trinitrophenyl-lipopolysaccharide.

borate and 0.08 M NaCl, pH 9.1) and incubating 18 h at 4°C. KLH beads were prepared by suspending esterified beads in 10 mg KLH/ml PBS. Beads suitable for adsorption of antigroup C carbohydrate antibodies were prepared by adding 75 μmol of the diazonium salt of the amino sugar (obtained by hydrogenation of *p*-nitrophenyl-α-D-2-acetamido-2-deoxygalactopyranoside) to 3 ml of BSA-coupled beads in 20 ml of BBS.

Antibodies in pooled rat immune sera were concentrated by Na_2SO_4 precipitation and separated into 7S and 19S fractions by gel filtration on Bio-Gel A-5m or AcA 34 columns (Bio-Rad Laboratories, Richmond, Calif.). The 7S fractions were passed through the appropriate prewashed immunoadsorbent column, and bound antibodies were eluted with 0.33 M acetic acid and neutralized immediately. The amount of eluted protein was estimated by OD_{280}, and the degree of purity was measured by the comparative binding of ^{125}I-trace-labeled protein (17) to appropriate and inappropriate immunoadsorbent beads; all isolated antibodies showed >70% specific binding.

Myeloma Proteins and Preparation of Anti-Subclass Antisera. Rat myeloma proteins IR595 (IgG_1), IR418 (IgG_{2a}), IR863 (IgG_{2b}), and IR309 (IgG_{2c}), used as standards in the radioimmunoassay procedure and for production of isotype-specific antisera, were purified from sera of tumor-bearing LOU/WsL rats as described previously (18). The rabbit anti-γ_1, goat anti-γ_{2a}, and rabbit anti-γ_{2c} were prepared as previously described by Bazin et al. (18). Rabbit anti-γ_{2b} obtained from Miles Laboratories, Inc., Miles Research Products, (Elkhart, Ind.) was rendered isotype specific by solid-phase absorption. The specificities of these reagents in the solid-phase radioimmunoassay procedure and/or facilitating antisera in the PFC assay are described in Results.

Radioimmunoassay. Concentrations of rat IgG subclasses were measured with inhibition-type solid-phase radioimmunoassays in which test samples were used to inhibit the binding of radiolabeled proband myeloma proteins to subclass-specific antisera (diluted 10- to 100-fold) which were covalently attached to esterified Sepharose 4B beads. 20 μl of 2–4% suspension of the beads in 1% bovine hemoglobin in PBS was added to 25 μl of test inhibitor protein, serially diluted in the same solution in 96-well microtiter plates. 20 μl of ^{125}I-labeled proband myeloma protein (1–10 ng, 2–20 μCi/μg) was added to each well. After an 18-h incubation in the cold, the beads were washed four times, dried in the bottom of the microtiter well, and counted in a gamma counter. Uninhibited binding of the proband was 10–15% of added counts; nonspecific binding was <0.5%. Each sample was run in triplicate, and each plate contained positive and negative controls. Subclass concentrations were standardized to total protein by reference to the inhibitory capacity of purified myeloma proteins.

Somatic Cell Hybrids. Hybrids were produced between mouse plasmacytoma cells (SP2/0-Ag14 or NSI/1-Ag4-1 [19]) and spleen cells from rats immune to DNP-OVA, GA-vaccine, and PC-KLH. 5 d after immunization, hybridization was performed essentially as described by Galfre et al. (20). Anti-DNP, anti-group A carbohydrate (GAC), and anti-PC-secreting hybrids were detected by ^{125}I-labeled antigen binding to isoelectric-focused culture supernates. Hybrids and monoclonal antibodies were cloned in soft agar (21) and maintained in tissue culture. Hybrids used in this study include the following: IgG_1 anti-DNP (49c-2), IgG_{2a} anti-DNP (50c-1), IgG_{2b} anti-GAC (148-d4), and IgG_{2c} anti-PC (120-a3).

Subclass-specific Plaque Assay. The subclass distribution of the splenic PFC IgG responses was measured by a subclass-specific hemolytic plaque assay (22). Sheep erythrocytes were coated with PC, GAC, and TNP determinants as described previously (23–25). OVA-erythrocytes were prepared by carbodiimide conjugation (26). Both direct and facilitated (IgG) PFC were measured. The specificity of the facilitating antisera was verified with the panel of hybridomas secreting the major rat immunoglobulin classes (Results). Facilitated splenic PFC were measured in the presence of 1/5,300 goat antimouse-μ (MOPC-104) to inhibit the appearance of all direct PFC. This concentration of anti-μ had no effect on facilitated IgG PFC.

Results

The specificities of the reagents used in this study are documented in Table II. In the radioimmunoassay, each subclass can be detected at concentrations <1 μg/ml

TABLE II

Specificity of Anti-Subclass Antisera

	Antiserum specificity			
	Anti-γ_1	Anti-γ_{2a}	Anti-γ_{2b}	Anti-γ_{2c}
Radioimmunoassay	Relative inhibition			
	I_{50} ($\mu g/ml$)			
Inhibitor				
IgG_1	0.04	>10	>10	>10
IgG_{2a}	>10	0.06	>10	>10
IgG_{2b}	>10	>10	0.20	>10
IgG_{2c}	>10	>10	>10	0.003
PFC assay	Facilitation of PFC			
	$PFC/10^5$ cells			
Hybridoma subclass				
IgG_1	2,770	<1	<1	<1
IgG_{2a}	<1	2,420	<1	<1
IgG_{2b}	<1	<1	1,660	<1
IgG_{2c}	<1	<1	<1	375

Anti-subclass antisera were tested for specificity by two different assays. First, the bead-absorbed antisera were exposed to radiolabeled myeloma proteins of the appropriate subclass and varying amounts of unlabeled myeloma proteins of all four IgG subclasses. Shown is the amount of protein ($\mu g/ml$) which inhibits 50% of the binding of the radiolabeled proband (I_{50}). Second, the antisera were tested for the ability to facilitate plaque-forming ability of hybridoma cells secreting antihapten antibodies. The specificity of the hybridoma antibodies is listed in Materials and Methods.

with 10^2- to 10^4-fold specificity. Similarly, the PFC assay detected each subclass with $>10^2$-fold specificity.

In preliminary studies, a variety of immunization protocols were tested using various antigens. Table I lists the protocols that generated sufficient IgG antibody responses for subclass analysis. For 8 of the 13 antigens, subclass analysis on both immunoadsorbent-purified serum antibodies and facilitated PFC was performed with essentially identical results. The data obtained by PFC analysis of antibodies stimulated by nine antigens and that obtained by analysis of purified serum antibodies elicited by the other four antigens are summarized in Fig. 1.

It is clear that rat antibody responses to the antigens studied here can be divided into three major categories: one category in which the antigens stimulate predominantly IgG_{2c} of the IgG subclasses, the second where both IgG_{2c} and IgG_{2b} predominate, and the third where IgG_{2c} is nearly absent and IgG_{2a} is dominant. An analogous division of these antigens can be made based on their IgG PFC responses in mice. Antigens that stimulate IgG_{2c} in rats stimulate IgG_3 in mice (7). These include PC-vaccine, GA-vaccine, DNP-Ficoll, and DNP-dextran, antigens that have been called thymus-independent type 2 (TI-2) based on responses in mice (27, 28). The second category includes three antigens that stimulate both IgG_{2b} and IgG_{2c} in rats and elicit IgG_2 and IgG_3 in mice (7). These include TNP-LPS and TNP-BA, classified as thymus-independent type 1 (TI-1) antigens (29), as well as GC-vaccine. Finally, all proteins and hapten-protein conjugates, except PC-KLH, elicit IgG_{2a} in the rat. These thymus-dependent antigens stimulate mainly IgG_1 in mice (7).

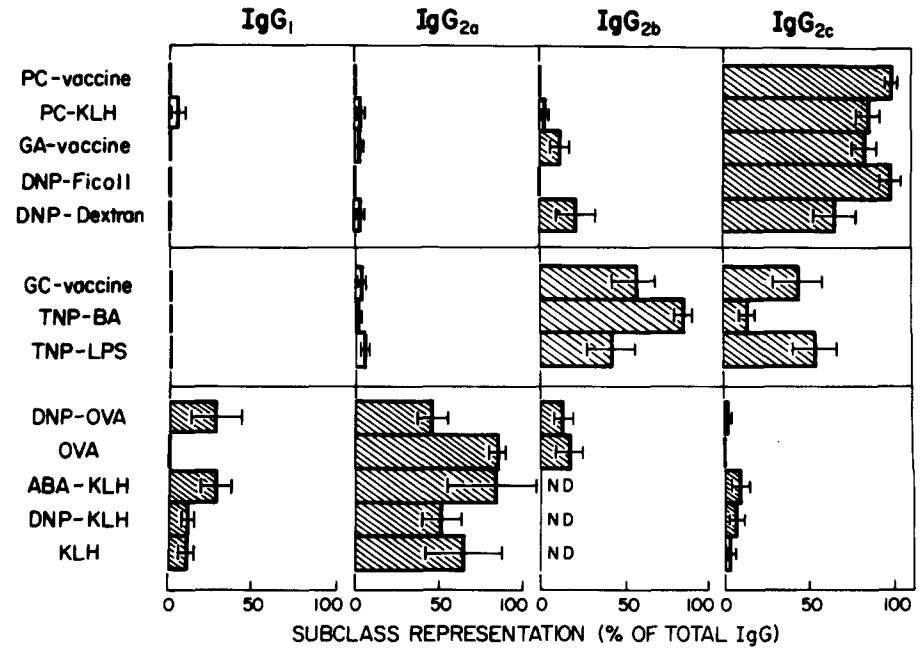

FIG. 1. IgG subclass distribution of rat antibodies to several antigens. Rats were immunized as described in Table I. Shown here are the geometric means and standard errors of the antigen-specific splenic PFC facilitated with anti-γ_1, -γ_{2a}, -γ_{2b}, and -γ_{2c} antisera, normalized to the total indirect PFC response. In the case of four antigens (Table I), analysis of the subclass composition of purified serum antibody was by inhibition-type radioimmunoassay.

If the four IgG isotypes appeared before rat-mouse divergence, then it is reasonable that antigens might stimulate analogous isotypes in both species. To further test for functionally analogous isotypes in the two species, the subclass patterns achieved in mice and those found in rats for nine antigens were plotted against one another (Fig. 2). Three significant correlations ($P \leq 0.005$) were found: mouse IgG_1 correlates with rat IgG_{2a}, mouse IgG_2 (a + b) correlates with rat IgG_{2b}, and mouse IgG_3 correlates with rat IgG_{2c}. The first two correlations may not be substantive because much of the correlation rests on lack of response in either species. Furthermore, failure to measure mouse IgG_{2a} and IgG_{2b} separately makes it impossible to draw conclusions concerning analogy with rat isotypes. However, the correlation between mouse IgG_3 and rat IgG_{2c}, shown in more detail in Fig. 3, appears highly significant because the comparison is based primarily on positive antibody responses of uncommon isotypes. The major exception to the correlation is PC-KLH, which stimulates IgG responses ~90% IgG_{2c} in rats and only ~25% IgG_3 in mice.

Discussion

Subclass restriction can occur potentially at two different levels: restriction in V_H and C_H gene pairing or selective expansion of only some subclasses from B cells with potential for expressing a random library of V_H-C_H combinations. Among the factors leading to selective expression could be T cell-mediated regulatory effects, or differing chemical makeup or form of the immunogen (e.g., carbohydrate vs. protein, soluble vs. particulate) possibly leading to different processing.

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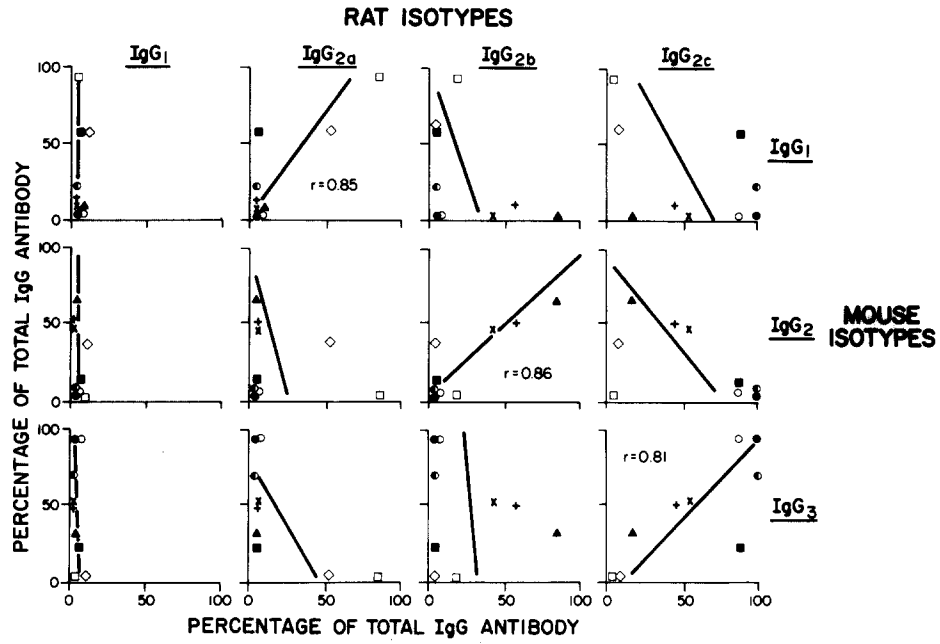

FIG. 2. Comparison of subclass distribution of mouse and rat antibodies elicited to nine antigens. Nine of the antigens listed in Fig. 1 have also been used in similar studies in mice (7): ●, PC-vaccine; ◐, DNP-Ficoll; ■, PC-KLH; ○, GA-vaccine; ×, TNP-LPS; +, GC-vaccine; ▲, TNP-BA; ◇, DNP-KLH; and □, OVA. Each of the 12 graphs represents a single mouse and a single rat IgG isotype plotting the percentage of each isotype stimulated by each of the nine antigens. In this way, functionally analogous isotypes may be found.

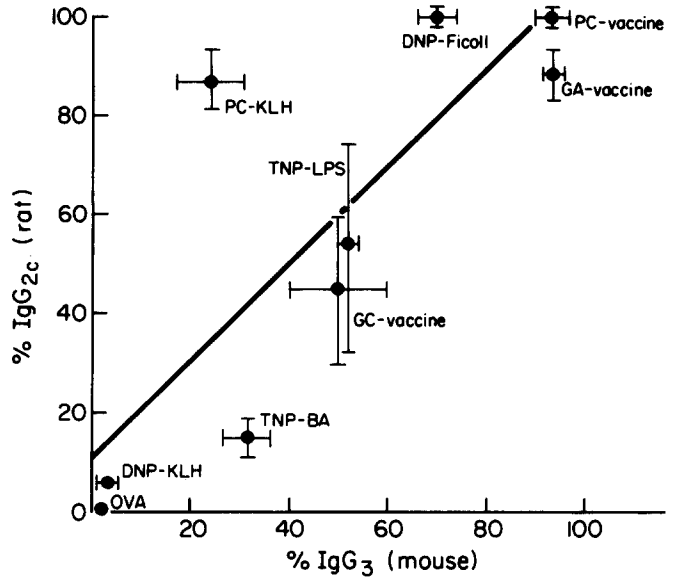

FIG. 3. Comparison of mouse IgG_3 and rat IgG_{2c} responses with the antigens shown in Fig. 2.

Numerous examples exist where adjuvant (30) or carrier determinants (31, 32) of the immunogen have been shown to alter the isotype of antibodies produced, but in few of these studies has it been determined whether the changes in isotype resulted from changes in variable regions expressed (33). In the present study, subclass restriction seems not to be dependent upon the nature of the antigen. Antigens that stimulate IgG_{2c} in the rat include bacterial carbohydrates (34), hapten-polysaccharide conjugates, or even hapten-protein conjugates (PC-KLH). These antigens have been administered either in complete Freund's adjuvant or in saline, as bacterial vaccines or soluble molecules. Thus, the ability to stimulate IgG_{2c} seems independent of adjuvant, route of immunization, or form of the antigen. Although the IgG_{2c}-stimulating antigens are largely carbohydrate, the exception of PC-KLH shows, at least, that IgG_{2c} restriction is not limited to carbohydrates. Furthermore, T cell function seems not to be the determining factor in subclass preference because ABA-KLH and DNP-KLH elicit IgG_{2a}, whereas PC-KLH stimulates IgG_{2c}. Similarly, antigens shown in other species to be thymus independent, like DNP-Ficoll (35) and PC-vaccine (23), and thymus dependent, like GA-vaccine (36) and PC-KLH, stimulate the same dominant subclass. Thus, no obvious attributes of the antigens or immunization protocols allow us to explain the subclass restriction.

Pertinent to the discussion of subclass preference by sets of antigens are results achieved with the CBA/N strain of mice. These animals have an X-linked deficiency in B lymphocyte development (37) that results in a relative failure to produce IgG_3 and IgM immunoglobulins (38). Thus, almost no antibodies are produced upon immunization with TI-2 antigens, only IgG_2 antibodies are produced to TI-1 antigens, and near normal responses result after immunization with thymus-dependent antigens (7). Further analysis showed at least two distinct subpopulations of B lymphocytes in normal mice, distinguishable by differentiation antigens (39, 40), surface immunoglobulin density (40), and age of development; one of these subpopulations is deficient in the CBA/N mouse. Furthermore, several studies have suggested that the different categories of antigens stimulate different subpopulations of B cells (41–43). These studies suggest, therefore, that there are different subpopulations of B lymphocytes, each responsive to different groups of antigens, and possibly each generating distinct subclasses of immunoglobulins.

This interpretation raises interesting questions concerning the interrelationships between the various B lymphocyte subpopulations. If the subpopulations represent different stages of differentiation of a single line of B lymphocytes (44), why are particular variable regions (e.g., anti-PC in rats) expressed predominantly in one subpopulation? On the other hand, the subpopulations may represent distinct lines of B cells restricted not only in C_H gene expression, but also in V_H gene expression as well (7). Clearly, much more work needs to be done to resolve these issues.

Finally, this study demonstrates that IgG_{2c} in the rat and IgG_3 in the mouse are most likely analogous subclasses. Both immunoglobulins are euglobulins, fix complement poorly, and, as we will show elsewhere,[1] share antigenic determinants. It will not be surprising if the rat, like the mouse, possesses a distinct B lymphocyte subpopulation restricted to IgG_{2c} production. Thus, at least IgG_3-IgG_{2c} subclasses arose before rat-mouse speciation. VanLoghem and Litwin (4), studying the distribution of Gm allotype markers among primates, concluded that IgG_2, the subclass associated with many anticarbohydrate variable regions, was the most primitive of

the human IgG subclasses. Whether human IgG_2 shares a common ancestry with mouse IgG_3 and rat IgG_{2c} remains a provocative question. It is likely, however, that continued study of the phenomenon of subclass restriction will lead to better understanding not only of immunoglobulin evolution, but also of variable region gene expression.

Summary

The IgG subclass distribution of rat antibodies to 13 different antigens was measured. Antibodies to protein and hapten-protein conjugates were predominantly IgG_{2a}. Antigens labeled thymus-independent type 1, based upon responses in mice, stimulated both IgG_{2b} and IgG_{2c} antibodies, but little IgG_{2a}. Polysaccharide and hapten-polysaccharide antigens (thymus-independent type 2) as well as phosphocholine-keyhole limpet hemocyanin, stimulated predominantly IgG_{2c} antibodies. A division of antigens into essentially the same categories has been made on the basis of subclass restriction in mice. Antigens that stimulate IgG_{2c} in rats stimulate IgG_3 in mice. Thus, by comparing subclass preference with a variety of antigens, functional analogues among subclasses in different species can be identified.

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