SUBCLASS RESTRICTION OF MURINE ANTIBODIES

III. Antigens That Stimulate IgG$_3$ in Mice

Stimulate IgG$_{2c}$ in Rats*

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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.

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 × 10⁸ 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 × 10⁹ 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.

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).
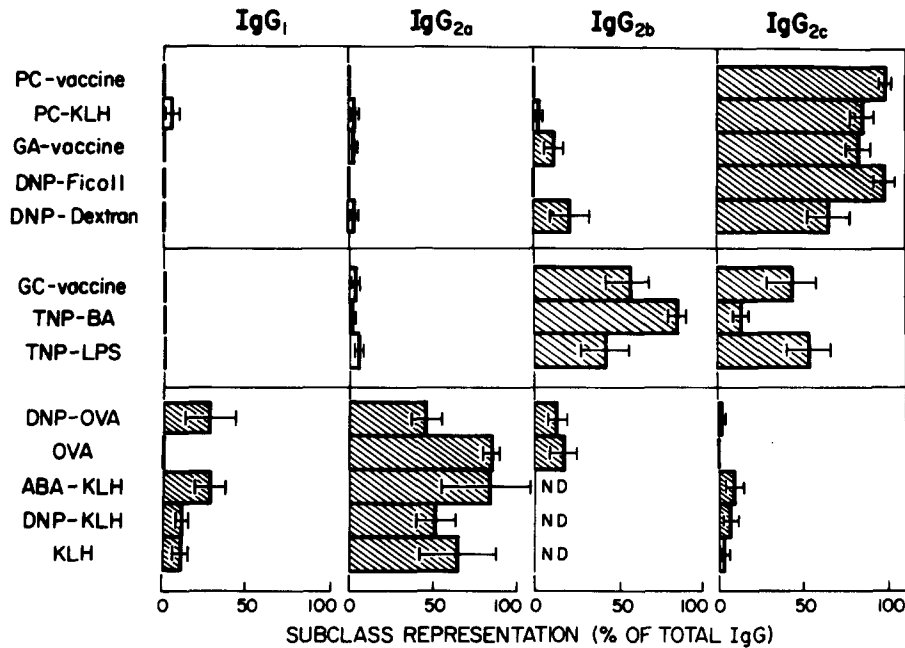
Fig. 1. IgG subclass distribution of rat antibodies to several antigens. Rats were immunized as described in Table I. Shown here are the geometric means and standard errors of the antigen-specific splenic PFC facilitated with anti-γ_1, -γ_{2a}, -γ_{2b}, and -γ_{2c} antisera, normalized to the total indirect 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.
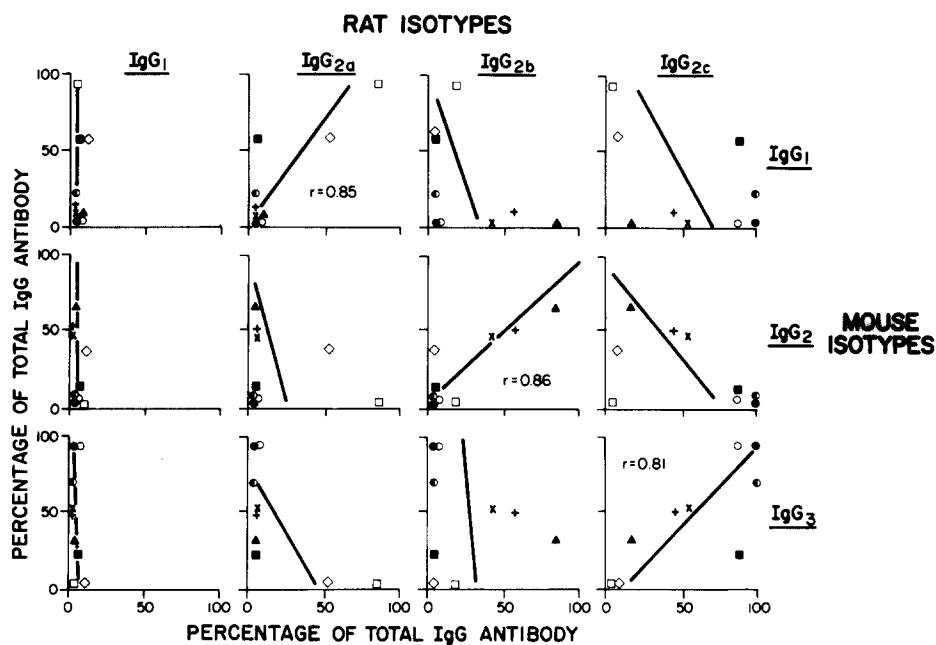
RAT ISOTYPES

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.
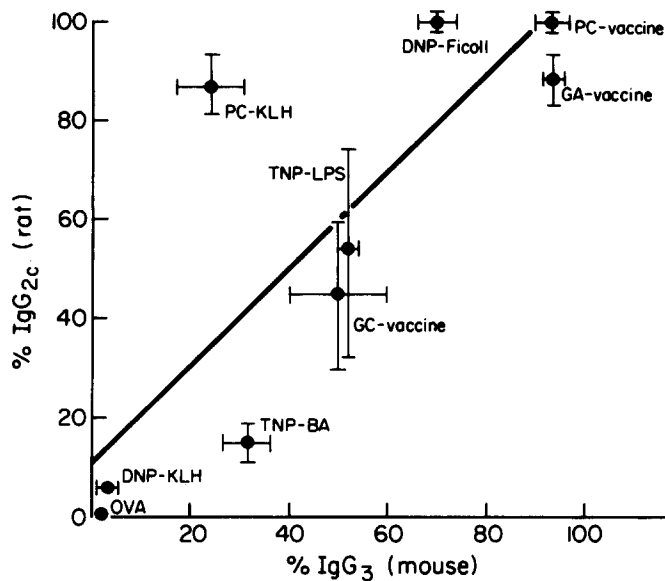

Fig. 3. Comparison of mouse IgG₃ and rat IgG₂c responses with the antigens shown in Fig. 2.

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.

References

1. Pink, J. R. L., S. H. Buttery, G. M. DeVries, and C. Milstein. 1970. Human immunoglobulin subclasses. Partial amino acid sequence of the constant region of a γ4 chain. *Biochem. J.* 117:133.

2. Cornell, G. E., D. M. Parr, and T. Hoffman. 1979. The amino acid sequences of the three heavy chain constant region domains of a human IgG2 myeloma protein. *Can. J. Biochem.* 57:758.

3. Brunhouse, R., and J. J. Cebra. 1979. Isotypes of IgG: comparison of the primary structures of three pairs of isotypes which differ in their ability to activate complement. *Mol. Immunol.* 16:907.

4. VanLoghem, E., and S. D. Litwin. 1972. Antigenic determinants on immunoglobulins of non-human primates. *Transplant. Proc.* 4:129.

5. Yount, W. J., M. M. Dorner, H. G. Kunkel, and E. A. Kabat. 1968. Studies on human antibodies. VI. Selective variations in subgroup composition and genetic markers. *J. Exp. Med.* 127:633.

6. Perlmutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 121:566.

7. Slack, J., G. P. Der-Balian, M. Nahm, and J. M. Davie. 1980. 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. *J. Exp. Med.* 151:853.

8. Grey, H. M., J. W. Hirst, and M. Cohn. 1971. A new mouse immunoglobulin: IgG$_3$. *J. Exp. Med.* 133:289.

9. Perlmutter, R. M., D. E. Briles, and J. M. Davie. 1977. Complete sharing of light chain spectrotypes by murine IgM and IgG anti-streptococcal antibodies. *J. Immunol.* 118:2161.

10. Inman, J. K. 1975. Thymus-independent antigens: the preparation of covalent, hapten-Ficoll conjugates. *J. Immunol.* **114**:704.

11. Little, J. R., and H. N. Eisen. 1967. Preparation of immunogenic 2,4-dinitrophenyl and 2,4,6,-trinitrophenyl proteins. *Methods Immunol. Immunochem.* **1**:128.

12. Mosier, D. E. 1978. Induction of B cell priming by neonatal injection of mice with thymic-independent (type 2) antigens. *J. Immunol.* **121**:453.

13. Jacobs, D M., and D. C. Morrison. 1975. Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). *J. Immunol.* **114**:360.

14. Chesebro, B., and H. Metzger. 1972. Affinity labeling of a phosphorylcholine binding mouse myeloma protein. *Biochemistry.* **11**:766.

15. Nisonoff, A. 1967. Coupling of diazonium compounds to proteins. *Methods Immunol. Immunochem.* **1**:120.

16. Cuatrecasas, P., and I. Parikh. 1972. Adsorbents for affinity chromatography. Use of *N*-hydroxysuccinimide esters of agarose. *Biochemistry.* **11**:2291.

17. Greenwood, G. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.

18. Bazin, H., A. Becker, and P. Querinjean. 1974. Three classes and four (sub)classes of rat immunoglobulins: IgM, IgA, IgE, and IgG1, IgG2a, IgG2b, IgG2c. *Eur. J. Immunol.* **4**:44.

19. Shulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.).* **276**:269.

20. Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigen produced by hybrid cell lines. *Nature (Lond.).* **266**:550.

21. Coffino, P., R. Baumal, R. Laskov, and M. D. Scharff. 1972. Cloning of mouse myeloma cells and detection of rare variants. *J. Cell. Physiol.* **79**:429.

22. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* **6**:588.

23. Claflin, J. L., R. Lieberman, and J. M. Davie. 1976. Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. *J. Exp. Med.* **139**:58.

24. Briles, D. E., and J. M. Davie. 1975. Clonal dominance. I. Restricted nature of the IgM antibody response to group A streptococcal carbohydrate in mice. *J. Exp. Med.* **141**:1291.

25. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.

26. Johnson, H. M., K. Brenner, and H. E. Hall. 1966. The use of a water-soluble carbodiimide as a coupling reagent in the passive hemagglutination test. *J. Immunol.* **97**:791.

27. Mosier, D. E., I. M. Zitron, J. J. Mond, A. Ahmed, I. Sher, and W. E. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. *Immunol. Rev.* **37**:89.

28. Mond, J. J., I. Scher, D. E. Mosier, M. Baese, and W. E. Paul. 1978. T-independent responses in B cell-defective CBA/N mice to *Brucella abortus. Eur. J. Immunol.* **8**:459.

29. Mosier, D. E., I. Sher, and W. E. Paul. 1976. In vitro responses of CBA/N mice: spleen cells of mice with an X-linked defect that precludes immune responses to several thymus-independent antigens can respond to TNP-lipopolysaccharide. *J. Immunol.* **117**:1363.

30. Benacerraf, B., Z. Ovary, K. J. Bloch, and E. C. Franklin. 1963. Properties of guinea pig 7S antibodies. I. Electrophoretic separation of two types of guinea pig 7S antibodies. *J. Exp. Med.* **117**:937.

31. Siskind, G. W., W. E. Paul, and B. Benacerraf. 1966. Studies on the effect of the carrier molecule on antihapten antibody synthesis. I. Effect of carrier on the nature of antibody synthesized. *J. Exp. Med.* **123**:673.

32. Liu, S. H., P. H. Koo, and J. J. Cebra. 1974. Effect of carrier priming on the distribution

of anti-hapten antibodies between IgG1 and IgG2 isotypes in the hyperimmune guinea pig. *J. Immunol.* **113:**677.

33. Schroer, J., and J. M. Davie. 1977. Functional, structural, and antigenic similarities of guinea pig anti-phosphorylcholine antibodies. *J. Immunol.* **118:**1987.

34. Leslie, G. A. 1979. Expression of a cross-reactive idiotype on the IgG2c subclass of rat anti-streptococcal carbohydrate antibody. *Mol. Immunol.* **16:**281.

35. Sharon, R., P. R. B. McMaster, A. M. Kask, J. D. Owens, and W. E. Paul. 1975. DNP-lys-Ficoll: a T-dependent antigen which elicits both IgM and IgG anti-DNP antibody-secreting cells. *J. Immunol.* **114:**1585.

36. Braun, D. G., B. Kindred, and E. B. Jacobson. 1972. Streptococcal group A antibodies in mice: evidence for strain differences in magnitude and restriction of the response and for thymus dependence. *Eur. J. Immunol.* **2:**138.

37. Paul, W. E., B. Subbarao, J. J. Mond., D. G. Sieckmann, I. Zitron, A. Ahmed, D. E. Mosier, and I. Scher. 1979. B lymphocyte development and activation: analysis with a mutant mouse strain. *In* Cells of Immunoglobulin Synthesis. B. Pernis and H. J. Vogel, editors. Academic Press, Inc., New York. 383.

38. Perlmutter, R. M., M. Nahm, K. E. Stein, J. Slack, I. Zitron, W. E. Paul, and J. M. Davie. 1979. Immunoglobulin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. *J. Exp. Med.* **149:**993.

39. Huber, B., R. K. Gershon, and H. Cantor. 1977. Identification of a B-cell surface structure involved in antigen-dependent triggering: absence of this structure on B-cells from CBA/N mutant mice. *J. Exp. Med.* **145:**10.

40. Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell. 1977. B-lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med.* **145:**101.

41. Lewis, G. K., and J. W. Goodman. 1977. Carrier-directed anti-hapten responses by B-cell subsets. *J. Exp. Med.* **146:**1.

42. Mosier, D. E., J. J. Mond, and E. A. Goldings. 1977. The ontogeny of thymic independent antibody responses *in vitro* in normal mice and mice with an X-linked B cell defect. *J. Immunol.* **119:**1874.

43. McKearn, J. P., and J. Quintans. 1979. Ontogeny of murine B-cell responses to thymus-independent trinitrophenyl antigens. *Cell. Immunol.* **44:**367.

44. Cambier, J. C., E. S. Vitetta, J. W. Uhr, and J. R. Kettman. 1977. B-cell tolerance. II. Trinitrophenyl human gamma globulin-induced tolerance in adult and neonatal murine B cells responsive to thymus dependent and independent forms of the same hapten. *J. Exp. Med.* **145:**778.