SUBCLASS RESTRICTION OF MURINE ANTIBODIES

III. Antigens That Stimulate IgGs in Mice
Stimulate IgG2a 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, IgG2, 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 IgG1. On the other hand, IgG antibodies to carbohydrates or hapten-carbohydrate conjugates are largely IgG3, 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 IgG3 in mice stimulate IgG2a in rats. Furthermore, as we will show elsewhere, these subclasses share antigenic determinants. 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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Antigens and Immunization. Keyhole limpet hemocyanin (KLH) 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 B 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-O-dextran B and DNP-O-FicolI) were prepared according to Inman (10) and conjugates with proteins (DNP-OA and DNP-O-KLH) according to Little and Eisen (11). Trinitrophenyl Brucella abortus (TNP-BA) and trinitrophenyl lipopolysaccharide (TNP-LPS) were prepared as before (12, 13). Phosphocholine (PC)-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 | PFC response | n Direct PFC/spleen | Indirect PFC/spleen |
|-------------|-----------------------|--------------|---------------------|---------------------|
| PC-vaccine  | 5 × 10⁶ R36a i.p.     | —            | —                   | —                   |
| PC-KLH      | 200 µg (CFA) i.p.     | 200 µg       | 5                   | 17,400 (1.5)        |
| GA-vaccine  | 30 µg rhamnose i.p.   | 30 µg        | 3                   | 63,400 (1.2)        |
| GC-vaccine  | 30 µg rhamnose i.v.   | 30 µg        | 6                   | Serum analysis only |
| DNP-O-FicolI| 200 µg i.p.           | —            | —                   | 12,500 (1.5)        |
| DNP-O-Dextran| 1,250 µg i.p.         | —            | —                   | 133,500 (1.5)       |
| DNP-O-OVA   | 200 µg (CFA) i.p.     | 200 µg       | —                   | 3,400 (1.5)         |
| OVA         | 200 µg (CFA) i.p.     | 200 µg       | —                   | <100 (1)            |
| ABA-KLH     | 300 µg (CFA) i.p.     | 250 µg       | 5                   | Serum analysis only |
| DNP-O-KLH   | 1,000 µg (CFA) i.p.   | 500 µg       | —                   | Serum analysis only |
| KLH         | 250 µg (CFA) i.p.     | 250 µg       | —                   | Serum analysis only |
| TNP-BA      | 5 × 10⁶ organisms     | —            | —                   | 27,200 (4.1)        |
| TNP-LPS     | 200 µg i.p.           | —            | —                   | 11,300 (2.2)        |

* 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 isotypes 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.

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, phosphocholine; PC-vaccine, Streptococcus pneumoniae strain R36A vaccine; PFC, plaque-forming cell(s); T1-1, thymus-independent type 1 antigen; T1-2, thymus-independent type 2 antigen; TNP-BA, trinitrophenyl-Brucella abortus; TNP-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 Na2SO4 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 immunoabsorbent column, and bound antibodies were eluted with 0.33 M acetic acid and neutralized immediately. The amount of eluted protein was estimated by Ols0, and the degree of purity was measured by the comparative binding of 125I-trace-labeled protein (17) to appropriate and inappropriate immunoabsorbent beads; all isolated antibodies showed >70% specific binding.

Myeloma Proteins and Preparation of Anti-Subclass Antisera. Rat myeloma proteins IR595 (IgG1), IR418 (IgG2a), IR863 (IgG2b), and IR309 (IgG2c), 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-γ1, goat anti-γ2a, and rabbit anti-γ2b were prepared as previously described by Bazin et al. (18). Rabbit anti-γ2b 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 125I-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 125I-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: IgG1 anti-DNP (49c-2), IgG2a anti-DNP (50c-1), IgG2b anti-GAC (148-d4), and IgG2c 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-γ1 | Anti-γ2a | Anti-γ2b | Anti-γ2c |
|-----------------------|---------|----------|----------|----------|
| Radioimmunoassay | Relative inhibition | \( I_{50} (\mu g/ml) \) |
| Inhibitor | | | | |
| IgG1 | 0.04 | >10 | >10 | >10 |
| IgG2a | >10 | 0.06 | >10 | >10 |
| IgG2b | >10 | >10 | 0.20 | >10 |
| IgG2c | >10 | >10 | >10 | 0.003 |
| PFC assay | Facilitation of PFC | \( PFC/10^9 \) cells |
| Hybridoma subclass | | | | |
| IgG1 | 2,770 | <1 | <1 | <1 |
| IgG2a | <1 | 2,420 | <1 | <1 |
| IgG2b | <1 | <1 | 1,600 | <1 |
| IgG2c | <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 (μg/ml) which inhibits 50% of the binding of the radiolabeled proband (150). 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 IgG2c of the IgG subclasses, the second where both IgG2a and IgG2b predominate, and the third where IgG2a is nearly absent and IgG2b is dominant. An analogous division of these antigens can be made based on their IgG PFC responses in mice. Antigens that stimulate IgG2c in rats stimulate IgG2c 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 IgG2b and IgG2c in rats and elicit IgG2 and IgG3 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 IgG2a in the rat. These thymus-dependent antigens stimulate mainly IgG1 in mice (7).
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 \( \sim 90\% \) IgG\(_{2c}\) in rats and only \( \sim 25\% \) 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.
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): 0, PC-vaccine; 0, DNP-Ficol; 0, PC-KLH; O, GA-vaccine; X, TNP-LPS; +, GC-vaccine; A, TNP-BA; O, DNP-KLH; and I-I, 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.

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

Fig. 2. Comparison of subclass distribution of mouse and rat antibodies elicited to nine antigens.

Fig. 3. Comparison of mouse IgGa 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 IgG₂ 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 IgG₂ seems independent of adjuvant, route of immunization, or form of the antigen. Although the IgG₂-stimulating antigens are largely carbohydrate, the exception of PC-KLH shows, at least, that IgG₂ 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 IgG₂, whereas PC-KLH stimulates IgG₂. 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 IgG₃ and IgM immunoglobulins (38). Thus, almost no antibodies are produced upon immunization with TI-2 antigens, only IgG₇ 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 C₃ gene expression, but also in V₃ gene expression as well (7). Clearly, much more work needs to be done to resolve these issues.

Finally, this study demonstrates that IgG₂ in the rat and IgG₃ in the mouse are most likely analogous subclasses. Both immunoglobulins are euglobulins, fix complement poorly, and, as we will show elsewhere, share antigenic determinants. It will not be surprising if the rat, like the mouse, possesses a distinct B lymphocyte subpopulation restricted to IgG₂ production. Thus, at least IgG₂-IgG₃ subclasses arose before rat-mouse speciation. VanLoghem and Litwin (4), studying the distribution of Gm allotype markers among primates, concluded that IgG₂, the subclass associated with many anticarbohydrate variable regions, was the most primitive of
the human IgG subclasses. Whether human IgG\textsubscript{2} shares a common ancestry with mouse IgG\textsubscript{2} and rat IgG\textsubscript{2a} 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\textsubscript{2a}. Antigens labeled thymus-independent type 1, based upon responses in mice, stimulated both IgG\textsubscript{2b} and IgG\textsubscript{2a} antibodies, but little IgG\textsubscript{2e}. Polysaccharide and hapten-polysaccharide antigens (thymus-independent type 2) as well as phosphocholine-keyhole limpet hemocyanin, stimulated predominantly IgG\textsubscript{2e} 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\textsubscript{2e} in rats stimulate IgG\textsubscript{2} 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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