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## Isolation of rat IgM to IgG hybridoma isotype switch variants and analysis of the efficiency of rat Ig in complement activation

Sequential sublining was used in combination with enzyme-linked immunosorbent assays to isolate  $\mu \rightarrow \gamma$  isotype switch variants of the rat IgM secreting mouse-rat B cell hybridoma line BA1.8. Switch variants to all four subclasses of IgG were obtained. The variant antibodies retained the antigen specificity of the parental IgM for the O18 (lipopolysaccharide) antigen of *Escherichia coli*. In sodium dodecyl sulfate-polyacrylamide gels the apparent molecular mass of the  $\gamma$  heavy chains decreased in the order  $\gamma_{2b} > \gamma_1 > \gamma_{2c} > \gamma_{2a}$ .

IgM, IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub> and IgG<sub>2c</sub> of the BA1.8 variant family and IgG<sub>2b</sub>, IgE and IgA of the previously described BA1.2 family were used for a comparative analysis of the capacity of rat Ig to activate complement. Efficient lysis of sheep erythrocytes coated with the O18 antigen was observed with IgM and all IgG subclasses, but no lysis was triggered by IgE or IgA. One hundred to 1000 IgG molecules were required to mediate the same hemolytic activity as one IgM molecule. The four IgG subclasses were equally efficient at mediating lysis by rat or human complement, while IgG<sub>2a</sub> was less efficient with guinea pig complement than the other three IgG subclasses.

Antibody-triggered binding of C3 to pathogenic O18:K1 *E. coli* bacteria was measured using serum containing <sup>125</sup>I-labeled C3. K1-encapsulated strains did not fix C3 efficiently in the absence of specific antibodies while acapsular mutants fixed C3 via the alternative pathway. IgM and all IgG subclasses triggered C3 binding to the K1 encapsulated bacteria. The capacity of IgM to mediate C3 fixation was not greater than that observed with IgG.

### 1 Introduction

The availability of monoclonal antibodies facilitates the systematic investigation of the efficiency with which different classes and subclasses of Ig exert their activities. In most former studies myeloma proteins of unknown antigen specificity or sets of Ig produced by independently derived hybridomas have been used. Because Ig effector functions are usually a consequence of antigen binding, studies with antibodies of unknown specificity or with sets of antibodies which differ in fine specificity or affinity may be of limited value.

Matched sets of Ig which have structurally identical variable region genes but different C<sub>H</sub> region genes can be obtained by the isolation of hybridoma Ig heavy chain class switch variants [1, 2]. Hybridoma mouse Ig heavy chain class switch variants occur with a frequency between 10<sup>-4</sup> and 10<sup>-10</sup> per cell generation [1, 3]. In most cases class-switching follows the order of the C<sub>H</sub> genes on the chromosome [3]. The gene order for the rat Igh locus has been determined as  $\mu$ - $\delta$ -( $\gamma_{2c}$ ,  $\gamma_{2a}$ )- $\gamma_1$ - $\gamma_{2b}$ - $\epsilon$ - $\alpha$  [4]. We have previously described the use of sequential sublining [3] in combination with enzyme-linked immunosorbent

assay (ELISA) for the isolation of rat Ig isotype switch variants [2]. Stable IgG to IgE and IgE to IgA variants were obtained, but all IgM to IgG variants detected in that study were relatively unstable, and yielded at a high frequency cells which failed to produce Ig heavy chains. In this report we describe the isolation of a complete set of stable switch variants of the IgM-producing hybridoma cell line BA1.8 to all four subclasses of IgG. It should be noted, that some authors could only obtain IgD variants from mouse IgM-producing hybridomas [1], while others also succeeded in the isolation of mouse IgM to IgG variants [5, 6]. In this report we also compare the capacity of antibodies of the BA1.2 (IgG<sub>2b</sub>, IgE, IgA) and the BA 1.8 (IgM, IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, IgG<sub>2c</sub>) families to mediate lysis of antigen-coated erythrocytes and to trigger C3 fixation to serum-resistant O18:K1 *E. coli* cells, which are a common cause of neonatal sepsis and meningitis [7].

### 2 Materials and methods

#### 2.1 Bacterial strains

Bacteria were grown in L broth at 37°C on a rotary shaker and harvested in exponential phase. The *E. coli* strains A56 (O18:K1), RS228 (O18:K1) and A76 (O7:K1) have been described in detail elsewhere [8]. The spontaneous acapsular mutants A56-10, A691 and A487 were selected for resistance to capsule-specific bacteriophage [8].

#### 2.2 Antisera

Rabbit antisera against the rat IgG subclasses [9] were kindly provided by Prof. H. Bazin (University of Louvain, Brussels, Belgium). Unlabeled and biotin-labeled affinity-purified goat

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**Abbreviations:** ELISA: Enzyme-linked immunosorbent assay  
mAb: Monoclonal antibody(ies) PBS: Phosphate-buffered saline  
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis LPS: Lipopolysaccharide

anti-rat IgG antibodies were obtained from Kirkegaard and Perry (Gaithersburg, MD), alkaline phosphatase-labeled goat anti-rabbit IgG antibodies and alkaline phosphatase-labeled streptavidin were obtained from Zymed (San Francisco, CA). Monoclonal mouse IgG<sub>1</sub> anti-rat IgG<sub>2b</sub> (MARG2b-8), IgG<sub>1</sub> anti-rat IgG<sub>2c</sub> (MARG2c-2) and IgG<sub>1</sub> anti-rat IgG<sub>1</sub> (MARG1-1) were obtained from Serotec (Blackthorn, GB). Monoclonal mouse IgG<sub>2b</sub> anti-rat IgG<sub>2a</sub> (RG 9/6.13) was obtained from Hybritech (San Diego, CA).

### 2.3 Isolation of hybridoma $\mu \rightarrow \gamma$ switch variants

Sequential sublining for the isolation of heavy chain switch variants was done as described [2, 3]. ELISA for the detection of IgG<sub>1</sub>, IgG<sub>2a</sub> or IgG<sub>2b</sub> was done as follows: ELISA plates (Dynatech, Kloten, Switzerland) were coated with affinity-purified goat anti-rat IgG antibodies [1  $\mu$ g/ml in phosphate-buffered saline (PBS)] overnight at 4°C. The wells were washed and incubated sequentially (a) with PBST [PBS containing 0.05% Tween 20 (Fluka AG., Buchs, Switzerland)] for 1 h at room temperature, (b) with hybridoma culture supernatants overnight at 4°C; (c) with biotin-conjugated goat anti-rat IgG antibodies (0.2  $\mu$ g/ml, preincubated in rat IgM-containing hybridoma culture supernatants to reduce IgM-related background) for 3 h at room temperature; (d) with alkaline phosphatase-labeled streptavidin [0.2  $\mu$ g/ml in PBSB (PBS containing 1% bovine serum albumin)]; and (e) with phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate, 0.02% MgCl<sub>2</sub> in sodium carbonate buffer, pH 9.6) for 1 to 4 h at room temperature. Between each step the plates were washed several times with PBST and water. The color change of the substrate solution was followed at 405 nm with a SLT210.1 ELISA reader (SLT Labinstruments, Grödig, Austria). Because the assay described above was too insensitive for IgG<sub>2c</sub>, rabbit anti-rat IgG<sub>2c</sub> antiserum (1:5000 in PBSB) was used in step (c) and alkaline phosphatase-labeled goat anti-rabbit IgG antibodies in step (d) for the detection of IgG<sub>2c</sub>.

### 2.4 Production of monoclonal antibodies

Hybridoma cells were cultured in Iscove's modified Dulbecco's medium (Gibco, Paisley, Scotland) as described [2]. Isolation of the O18 lipopolysaccharide (LPS)-specific rat-mouse hybridomas BA1.8 (IgM), BA1.2 (IgG<sub>2b</sub>), BA1.2E (IgE) and BA1.2A (IgA) has been described [2]. Antibodies were purified by column affinity chromatography using an O18 LPS-immunosorbent (BA1.8 family) or a rhamnose-immunosorbent (BA1.2 family). Coupling of rhamnose or LPS to epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) was done according to the manufacturer's description. Antibodies were eluted with 3 M sodium thiocyanate and dialyzed extensively. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Biosynthetic labeling of Ig and SDS-PAGE was done as described [2].

### 2.5 Passive hemolysis and hemagglutination

Sheep blood was collected in an equal volume of Alsever's solution and stored at 4°C for more than 7 and less than 30 days. The erythrocytes were washed three times with PBS

before use and coated with a saturating amount of O18 LPS (30  $\mu$ g LPS per 200  $\mu$ l packed volume of erythrocytes) for 45 min at 37°C with frequent agitation. LPS consisted of long-chain molecules prepared according to Neibert, M. and Achtmann, M. (manuscript in preparation). Briefly, LPS isolated by the hot phenol-water method [10] was treated with 0.1 M NaOH at 60°C for 12 min. Thereafter the long-chain molecules were purified by gel filtration in Tris deoxycholate and were subjected to dialysis, lyophilization and electro dialysis against triethylamine. Coated erythrocytes were washed with PBS three times and resuspended in hemolysis buffer (5 mM sodium Veronal, 145 mM NaCl, 0.15 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, pH 7.3). Passive hemolysis was performed in V-shaped microtiter plates (Nunc, Roskilde, Denmark) containing 0.4  $\mu$ l of packed coated erythrocytes, mAb diluted in hemolysis buffer and serum as a complement source in a total volume of 125  $\mu$ l. The final serum concentrations used were 1% (guinea pig serum or serum from germ-free outbred rats) or 3% (human serum absorbed with coated erythrocytes). After 1 h of incubation at 37°C, the plates were centrifuged and the supernatants transferred to flat-bottom microtiter plates which were scanned with a photometer at 405 nm. The amount of antibody per well sufficient for 50% hemolysis was calculated by interpolation of the dilution series.

For hemagglutination assays, the coated erythrocytes were incubated with serial dilutions of mAb in the absence of a complement source. The lowest amount of antibody that still yielded agglutination was determined.

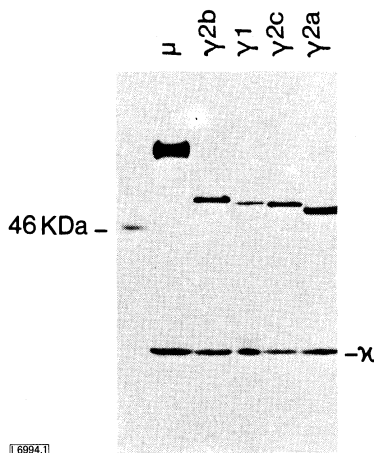
### 2.6 C3 binding to bacterial cells

Human C3 was purified to homogeneity as described [11] and was radiolabeled using IODO-GEN (Pierce Chemical Co., Rockford, MD). Specific activities of <sup>125</sup>I-C3 ranged from 5  $\times$  10<sup>5</sup> to 9  $\times$  10<sup>5</sup> cpm/ $\mu$ g. Incubation mixtures contained 20% germ-free rat serum, 4  $\times$  10<sup>8</sup> bacterial cells, 3  $\mu$ g <sup>125</sup>I-C3, mAb and buffer (2.5 mM sodium Veronal, 72 mM NaCl, 0.15 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.05% gelatin, 2.5% glucose, pH 7.3) in a total volume of 0.5 ml. After 15 min at 37°C, the bacterial cells were washed three times with ice-cold PBS and cell-associated radioactivity was monitored. The specific activity of C3 was calculated by dividing the radioactivity added by the total number of C3 molecules (human + rat), using a value for C3 concentration in rat serum of 3 mg/ml. Nonspecific binding which was measured by incubation with heat-inactivated (56°C, 30 min) serum was subtracted.

## 3 Results

### 3.1 Isolation of hybridoma isotype switch variants

For the isolation of  $\mu \rightarrow \gamma$  switch variants, the rat IgM-producing cell line BA1.8 was cultured in 1000 aliquots of 1000 cells each and IgG-producing subpopulations were identified by ELISA screening of the culture supernatants. A similar procedure was repeated with positive sublines until it was possible to clone the IgG-producing variants. IgG subclasses were identified with sets of subclass-specific monoclonal and polyclonal reagents. Switch variants of the cell line BA1.8 to all four subclasses of rat IgG were obtained. The variant antibodies retained the antigen specificity for the O18 antigen of *E. coli*. According to Rousseaux and Bazin [12] the molecular masses



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Figure 1. Analysis of variant Ig of the BA1.8 family by SDS-PAGE and autoradiography. Purified [<sup>35</sup>S]methionine-labeled Ig were reduced and subjected to electrophoresis on 10% polyacrylamide gels. The molecular mass marker protein was ovalbumin (46 kDa).

of rat Ig heavy chains are: μ: 72 kDa; γ<sub>1</sub> and γ<sub>2b</sub>: 55 kDa; γ<sub>2a</sub> and γ<sub>2c</sub>: 50 to 52 kDa. The heavy chains of the different Ig variants described in this report exhibited the expected size upon SDS-PAGE (Fig. 1). The molecular mass of the γ subclasses decreased in the order γ<sub>2b</sub> > γ<sub>1</sub> > γ<sub>2c</sub> > γ<sub>2a</sub>, whereas the κ light chains were indistinguishable from one another (Fig. 1). In addition to the BA1.8 variants, antibodies of the previously described BA1.2 family were also used for the analyses described below.

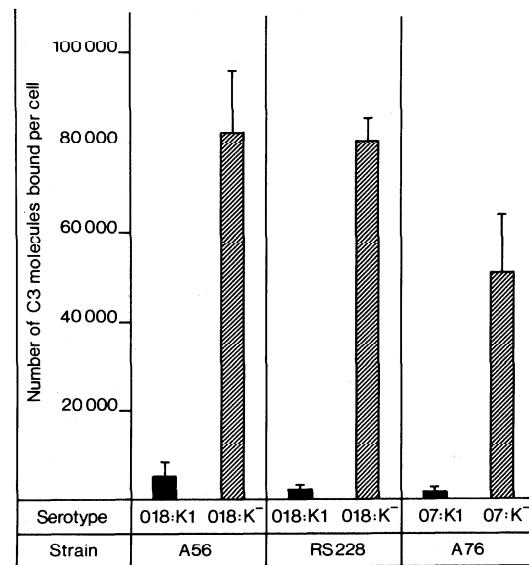
### 3.2 C activation

The antibodies of the BA1.2 and BA1.8 families were tested by agglutination and C-mediated lysis of O18 LPS-coated sheep erythrocytes. The three antibodies of the BA1.2 family (IgG<sub>2b</sub>, IgE and IgA) possessed a comparable specific hemagglutination activity (Table 1), but whereas IgG<sub>2b</sub> activated C efficiently, no hemolysis was observed with IgA or IgE (Table 1). With the BA1.8 family the IgM was about 10-fold more efficient than its IgG variants at promoting hemagglutination (Table 1) and 100-1000 times more efficient at promoting C-mediated lysis (Table 1). The four subclasses of IgG did not differ from each other in the ability to activate rat or

human C, but IgG<sub>2a</sub> was about ten times less efficient than the other three IgG subclasses with guinea pig C (Table 1).

### 3.3 C3 deposition on *E. coli* cells

Three serum-resistant K1 *E. coli* strains and their isogenic capsule-deficient mutants were tested for their ability to fix C3. Radioactivity associated with the bacterial cells after 15 min of incubation with rat serum containing <sup>125</sup>I-labeled human C3 was monitored and the number of C3 molecules bound per bacterial cell was calculated. All three capsule-deficient mutants fixed C3 very efficiently, while C3 fixation by the encapsulated bacteria was low (Fig. 2). Comparable results were obtained with Mg<sup>2+</sup>-EGTA-treated human or rat serum and with C4-deficient guinea pig serum (data not shown). The latter results indicate, that the alternative C pathway is the primary mechanism of C activation and of C3 fixation by the nonencapsulated *E. coli* bacteria. The K1 capsule,



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Figure 2. Number of C3 molecules fixed to K1 encapsulated *E. coli* bacteria and acapsular mutants, after 15 min of incubation in 20% rat serum. The data shown are the mean ± SE of the mean of three separate experiments.

Table 1.

Antibody	Hemagglutination activity <sup>a)</sup> (ng)	Hemolytic activity <sup>b)</sup> (ng)		
		Rat serum	Human serum	Guinea pig serum
IgM (BA1.8)	5-10	0.8-1.6	0.1-0.2	0.2-0.4
IgG <sub>1</sub> (BA1.8G1)	100-200	100-200	100-200	100-200
IgG <sub>2a</sub> (BA1.8G2a)	50-100	100-200	200-400	1000-2000
IgG <sub>2b</sub> (BA1.8G2b)	100-200	100-200	100-200	200-400
IgG <sub>2c</sub> (BA1.8G2c)	100-200	100-200	100-200	100-200
IgG <sub>2b</sub> (BA1.2)	50-100	100-200	50-100	100-200
IgA (BA1.2A)	25-50	>10000	>10000	>10000
IgE (BA1.2E)	25-50	>10000	>10000	>10000

- a) Minimal amount of antibody required for hemagglutination.
- b) Amount of antibody required for 50% hemolysis.

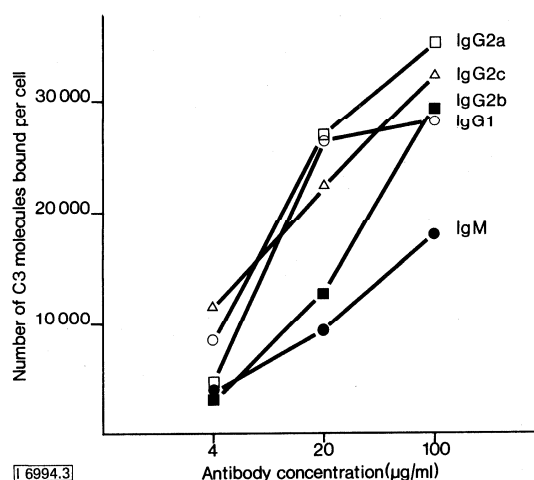


Figure 3. Antibody-mediated deposition of C3 molecules to O18:K1 *E. coli* cells. Bacteria were incubated for 15 min in 20% rat serum containing different amounts of antibodies of the BA1.8 family.

which is a sialic acid homopolymer seems to impede activation of the alternative pathway [8], similar to other sialic acid-containing cell surface structures [13].

The effect of anti-O18 Ig on the fixation of C3 by O18:K1 *E. coli* cells was also tested. It is remarkable that, based on the concentration of Ig molecules in the assay mixture, BA1.8 IgM was not more efficient than the IgG switch variants (Fig. 3). Antibodies of the BA1.2 family differed strongly in the capacity to mediate C3 deposition. The IgG<sub>2b</sub> BA1.2 was as efficient as the four BA1.8 IgG variants, but IgA and IgE had no significant activity (data not shown).

#### 4 Discussion

Isotype switch variant families are ideal tools for the analysis of heavy chain effector functions independently of differences in binding specificity or affinity. Here we have concentrated on the differential capacity of different classes and subclasses of rat Ig to activate C. The capacity of aggregated rat myeloma proteins of different IgG subclasses to consume C has been analyzed by Füst et al. [14]. Rat C consumption was observed with all four subclasses, while IgG<sub>2a</sub> failed in the consumption of human C and IgG<sub>1</sub> failed to consume guinea pig C [14]. Medgyesi et al. used polyclonal rat IgG preparations in the form of antigen-antibody complexes for similar analyses [15]. Complexes containing IgG<sub>1</sub> displayed a low capacity to activate rat C when compared with those containing IgG<sub>2a</sub>, IgG<sub>2b</sub> or IgG<sub>2c</sub> [15]. Hughes-Jones et al. investigated the mechanism of synergistic C-mediated lysis of rat erythrocytes using rat anti-class I antibodies and found that rat IgG<sub>2b</sub> has a stronger affinity for both guinea pig and human C1q than the other IgG subclasses [16]. The four different IgG subclasses of the BA1.8 family did not differ in the capacity to activate human or rat C while IgG<sub>2a</sub> was less efficient with guinea pig C than the other three subclasses. IgM was far more efficient than IgG in mediating lysis of antigen-coated erythrocytes and no lysis was observed with IgE and IgA.

Mouse Ig switch variants have been used to analyze the C activating capacity of mouse Ig [17, 18]. IgG<sub>1</sub> had a lower capacity to fix C than the other IgG subclasses [17, 18]. A

matched set of human immunoglobulins was recently tested for their ability to lyse hapten-coupled human red cells in the presence of human C [19]. Here IgG<sub>2</sub>, IgG<sub>4</sub> and IgE did not mediate lysis, whereas IgM, IgG<sub>1</sub> and IgG<sub>3</sub> were effective [19]. The structural basis for the wide variation in reactivity of different antibodies with homologous and heterologous C is only incompletely understood [20] and remains to be investigated further.

Conflicting results with respect to the relative protective efficiency of IgG and IgM against different invasive bacteria have been obtained [21-24]. A drawback of these studies is that antibodies which do not have identical variable regions were used. The O18-specific antibodies of the BA1.2 and BA1.8 families will allow an unambiguous comparative analysis of the prophylactic and therapeutic activities of Ig classes and subclasses in an experimental O18:K1 *E. coli* sepsis model [25, 26]. C3 opsonisation is one important defense mechanism against a variety of bacterial pathogens. The finding that IgM, although far more efficient than IgG in effecting lysis of antigen-coated red cells, was not more efficient than IgG in mediating C3 fixation to K1 *E. coli* cells is highly significant for the differential capacity of these antibodies to protect newborn rats against K1 *E. coli* infection (manuscript in preparation).

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