Bactericidal and Opsonic Activity of IgG1 and IgG2 Anticapsular Antibodies to *Haemophilus influenzae* Type b

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Complement-mediated bactericidal and opsonic activity of IgG1 and IgG2 antibodies to *Haemophilus influenzae* type b polysaccharide (polyribosyl ribitol phosphate [PRP]) were investigated. The antibody sources were IgG1 or IgG2 subclass polyclonal antibody fractions prepared by immunoabsorption of sera from adults immunized with PRP or PRP-diphtheria toxoid conjugate vaccine or clonally purified anti-PRP antibodies from eight adults immunized with PRP vaccine. In bactericidal assays using an inoculum of 3×10^3 colony-forming units (cfu)/ml, twofold lower concentrations of IgG1 compared with IgG2 antibody were required for 50% killing. With $\sim 10^6$ cfu/ml, IgG1 antibody killed 3 logs more of bacteria than were killed by comparable concentrations of IgG2 antibody. The IgG1 antibody also required lower concentrations of complement than did the IgG2 antibody for comparable bacteriolytic activity. Clonally purified IgG1 and IgG2 anti-PRP antibodies from most individuals showed similar relative differences in bactericidal activity. IgG1 anti-PRP antibody was also more efficient than IgG2 anti-PRP antibody in enhancing the uptake of radiolabeled type b *H. influenzae* by human polymorphonuclear leukocytes in the presence of complement and in protecting infant rats from developing bacteremia. However, the differences in opsonic or protective activity of the two subclasses were smaller than the differences in bactericidal activity. Thus, IgG1 anti-PRP antibody is functionally more effective than IgG2 antibody, but it is likely that both subclasses can confer protection against disease.

Serum antibodies to the capsular polysaccharide (polyribosyl ribitol phosphate [PRP]) of *Haemophilus influenzae* type b (Hib) confer immunity to invasive Hib disease. The isotype composition of serum antibody evoked in most adults and children >2 years of age immunized with PRP vaccine includes a combination of IgM, IgA, and IgG [1–3]. In most individuals the IgG antibody subclass consists of both IgG1 and IgG2, although a few individuals show a restricted IgG1 or IgG2 response [4, 5]. In contrast, the IgG antibody response of infants <18 months of age immunized with PRP-protein conjugate vaccines is usually restricted to IgG1 [6–8]. Only a restricted number of IgG antibody clones are demonstrated in response to PRP or PRP-protein conjugate vaccines [4, 9, 10]. Further, recent studies have shown that anti-PRP antibody V regions may share cross-reactive idiotypes [11, 11a] and use V_HIII and four different types of V_L gene families [12].

The functional activity of anti-PRP antibodies was characterized by Johnston et al. in 1973 [1] and later by Schreiber et al. in 1986 [13]. In the presence of complement, IgG antibodies were reported to have both bactericidal and opsonic activity. IgM antibody had only bactericidal activity, and IgA was neither bactericidal nor opsonic [13]. Tarr et al. in 1982 [14] and Steele et al. in 1984 [15] reported that IgG antibody to PRP activated bacteriolysis by both the classic and alternative complement pathways. Little information is available on the functional activity of anti-PRP antibody of different IgG subclasses. A study reported in 1986 prepared IgG1- and IgG2-depleted fractions from a serum IgG pool derived from healthy adult donors immunized with PRP vaccine [16]; no significant differences were found in the concentrations of IgG1 or IgG2 anti-PRP antibodies required to activate complement-mediated lysis of Hib cells in vitro or to confer protection against bacteremia in experimentally infected infant rats. However, the results of this study were limited to investigation of IgG1- and IgG2-depleted IgG subclass fractions. Thus, one could not exclude an effect of antibodies directed at noncapsular antigens. Also, because pooled IgG from multiple donors was used, differences in the activity of subclass-specific antibodies from individual donors might have been masked. In addition, the relative functional activity of antibodies with different defined V regions was not studied. Finally, opsonic activity, which may be more important than bactericidal activity in conferring protection against Hib disease, was not tested. Such information is important because the IgG sub-

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class composition or V region of the anti-PRP antibody response may vary with age and vaccine type.

The purposes of the present study were to compare complement-mediated bactericidal and opsonic activity of affinity-purified IgG1 anti-PRP antibody with that of IgG2 anti-PRP antibody, using preparations that allowed us to exclude the interaction of antibodies directed at noncapsular antigens, and to compare the functional activity of clonally purified IgG1 and IgG2 antibodies purified from sera of individual donors immunized with PRP vaccine.

Materials and Methods

Preparation of IgG pool. Three adults were injected subcutaneously with 25 μg of PRP vaccine (Praxis Biologics, Rochester, NY); one adult was immunized with PRP-diphtheria toxoid conjugate vaccine (PRP-D; Connaught Laboratories, Swiftwater, PA) containing 25 μg of PRP per dose. The sera that contained >40 μg/ml of total anti-PRP antibody were obtained 1–24 months later. The IgG1 antibody concentrations of the sera from the three adults vaccinated with PRP vaccine were 3.9, 60, and 116 μg/ml, and the respective IgG2 antibody concentrations were 36, 114, and 25 μg/ml. The serum from the adult given conjugate vaccine had about equal concentrations of IgG1 and IgG2 antibody (18 and 14 μg/ml, respectively). IgG fractions were prepared by gel filtration of Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). An aliquot of serum (~14 ml) was applied to the Sephacryl S-300 column (7.0 cm^2 × 72 cm; total volume, 500 ml), and chromatography was performed at room temperature in phosphate-buffered saline (PBS), pH 7.2, with 0.02% sodium azide. The anti-PRP antibody in the fractions was determined by class-specific ELISA [17]. IgG fractions that had no detectable IgA anti-PRP antibodies were pooled and concentrated by ultrafiltration with a PM 30 Diaflo filter (Amicon, Danvers, MA).

The concentrations of anti-PRP in the IgG fractions were measured by a radioantigen binding assay (RABA). Different volumes of IgG from each individual were then pooled so that each individual contributed ~25% of the anti-PRP antibodies to the IgG pool. The final IgG pool contained 2% IgA and <0.7% IgM as determined by nephelometry. The IgG subclasses consisted of IgG1 (396 mg/dl), IgG2 (250 mg/dl), IgG3 (7.5 mg/dl), and IgG4 (13.5 mg/dl) as determined by a particle concentration fluorescence immunoassay [18]. The anti-PRP antibody concentration was 55 μg/ml as determined by RABA.

Purification of anti-PRP antibody by affinity chromatography. To prepare IgG affinity-purified anti-PRP, a portion of the IgG pool was reacted with an affinity matrix containing Hib capsular polysaccharide. PRP was prepared according to the method of Kuo [19] and coupled to aminohexyl sepharose 4B (Pharmacia) using cyanogen bromide as described previously [15]. The affinity-purified anticapsular antibody was eluted with 3.5 *M* $MgCl_2$ and immediately diluted 1:2 in veronal-buffered saline (VBS) with 0.5% bovine serum albumin (BSA). The VBS buffer had a pH of 7.4 and 1 liter contained 8.5 g of NaCl, 0.46 g of sodium salt of barbital, and 0.51 g of barbituric acid. The eluted antibody was dialyzed against VBS buffer and the affinity-purified antibody, designated "IgG affinity-purified anti-PRP antibody" (figure 1), was concentrated to about the original concentration of anti-PRP antibodies in the IgG pool and filter-sterilized.

Purification of IgG1 and IgG2 anti-PRP antibodies. IgG1 and IgG2 fractions were prepared from the IgG affinity-purified anti-PRP antibody using affinity columns for human IgG1 and IgG2 (figure 1). In brief, monoclonal antibodies (MAb) HG11 (for IgG1) and HP6016 (for IgG2) were coupled to Sepharose 4B [4, 20]. Part of the IgG affinity-purified anti-PRP antibody pool was passed over the column specific for IgG1, and the rest was passed over the anti-IgG2 column. The fall-throughs were collected and designated IgG anti-PRP depleted of IgG1 antibody and IgG anti-PRP depleted of IgG2 antibody, respectively (fractions I and III, figure 1). The columns then were washed extensively with PBS, and the subclass fractions were eluted with 3 ml of 0.1 *M* glycine HCl, pH 2.6, and collected into 300 μl of 1 *M* Tris buffer, pH 7.5. The eluates were immediately dialyzed with PBS. The fractions were designated affinity-purified IgG1 and IgG2 anti-PRP antibodies, respectively (fractions II and IV, figure 1). Purity of these fractions was monitored by the capture ELISA for total IgG1, IgG2, and IgG4 as described below.

The remaining portion of the original IgG pool was divided into two parts and each was passed directly over the anti-IgG1 or anti-IgG2 columns five times as described (figure 1). The fall-throughs of the fifth pass, containing both anti-PRP and antinoncapsular an-

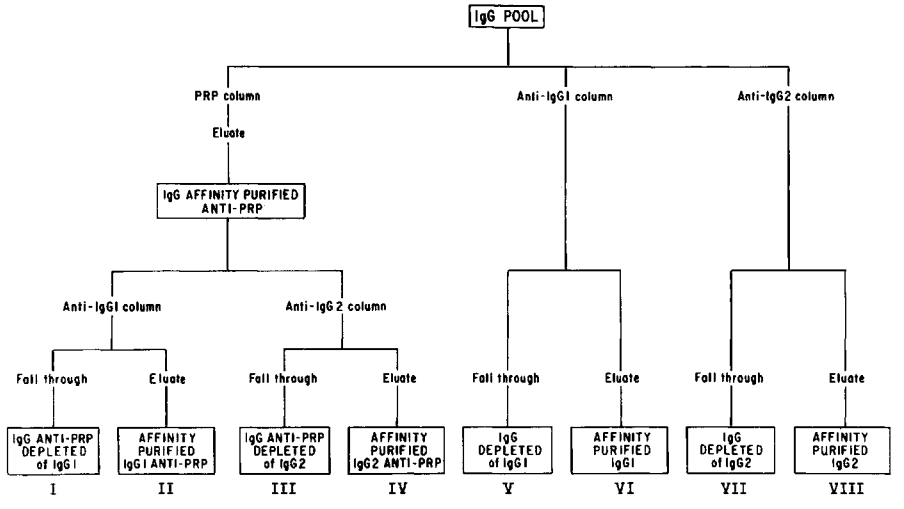

Figure 1. Flow chart describing preparation of human subclass fractions (I–VIII). IgG pool used as starting material was prepared by chromatography of postvaccination serum pool on Sephacryl S-300.

tibodies, were collected and designated IgG depleted of IgG1 or IgG2 fractions, respectively (fractions V and VII). After each pass, the subclasses were eluted as described, and all five eluates of each respective column were pooled. The eluates were designated affinity-purified IgG1 and IgG2, respectively (fractions VI and VIII).

Purification of clonal IgG1 and IgG2 anti-PRP antibodies from individual donors. Clonal anti-PRP antibodies were purified from the serum of eight adult volunteers immunized with 25 μg of PRP vaccine (Praxis) as described previously [10]. Selection of the donors was based on a high antibody response, and the donor identification used here is identical to that used previously [10]. Briefly, purified anti-PRP antibodies were obtained from 300–500 ml of postvaccine serum using PRP-specific affinity chromatography. Affinity-purified anti-PRP antibodies were separated into IgG1 and IgG2 fractions using affinity chromatography consisting of a MAb for IgG1 (HG11) or IgG2 (HP6016) coupled to Sepharose 4B [4, 10, 20]. IgG1 and IgG2 anti-PRP preparations were >99% pure by IgG subclass specific immunoassay [18].

Although IgG1 fractions were clonal with the exception of one individual (subject K), the IgG2 fractions were not [10]. Therefore, IgG2 anti-PRP antibodies from three individuals in this study were separated into clonal antibodies by a preparative modification of a previously described isoelectric focusing procedure [4, 10, 20]. This procedure allowed separation of the IgG2κ and IgG2λ antibodies from these individuals. All samples were dialyzed against VBS, filter-sterilized, and coded for bactericidal assay.

Serologic assays. The concentrations of total anti-PRP antibody were measured by a RABA as previously described [17, 21]. ^{125}I-PRP was used as the test antigen. A previously described solid-phase ELISA was used to measure IgG and IgA anti-PRP antibody [17]. The IgG and IgA were detected by biotin conjugates of γ-chain-specific goat anti-human IgG antibody or α-chain-specific goat anti-human IgA antibody (Tago, Burlingame, CA) diluted 1:5000 and 1:2500, respectively.

A capture ELISA was used to measure total IgG1, IgG2, or IgG4. In brief, Immulon 2 flat-bottom microtiter plates (Dynatech, Chantilly, VA) were coated with 10 μg/ml MAb (120 μl) for IgG1 (HG11), IgG2 (HP6016), or IgG4 (HP6023). Uncoated sites were blocked with 1% BSA diluted in PBS and 0.5% Tween 20. Appropriately diluted subclass fractions were added and titrated in serial fourfold dilutions and incubated at room temperature for 3 h. After these were washed, bound IgG subclasses were detected with alkaline phosphatase–conjugated goat anti-human IgG and *p*-nitrophenylphosphate as the substrate. IgG subclass concentrations in the unknown samples were calculated by comparing the absorbance of the unknown with that of a set of purified myeloma proteins and dilutions of a standard serum pool [18].

Bactericidal assay: high-inoculum type. The high-inoculum bactericidal assay was performed as described by Musher et al. [22], with the exception that the buffer consisted of VBS-BSA instead of Hanks' balanced salt solution with 0.1% gelatin used in the previous study. The reaction mixture (120 μl) comprised IgG subclass fractions at a final anti-PRP antibody concentration of 1 μg/ml as determined by RABA, 20% human agammaglobulinemic serum as a complement source [15], ~10^6 colony-forming units (cfu)/ml log-phase Hib cells (strain Minnesota A) [23], 0.15 m*M* $CaCl_2$, and 0.50 m*M* $MgCl_2$. The reaction vials were incubated at 37°C in a shaking water bath for 60 min, and serial dilutions were plated onto chocolate agar plates. The results were expressed as the log decrease in Hib cells after a 60-min incubation. Positive controls in each assay consisted of 10% and 0.5% of immune sera pool; negative controls consisted of complement source only and the IgG pool tested in the presence of 20% heat-inactivated complement.

Bactericidal assay: low-inoculum type. The low-inoculum bactericidal assay was performed as previously described [16]. The assay was similar to the high-inoculum assay except that instead of testing a single antibody concentration, the low-inoculum assay tested serial two-fold dilutions of antibody in the presence of 20% complement and an inoculum of ~3×10^3 cfu/ml log-phase Hib cells. The percentage of survival of Hib cells after incubation in the assay mixture was calculated from colony counts at 0 and 60 min. The results were expressed as the antibody concentration needed for 50% killing divided by the colony counts present in 1 ml. In this assay, in the absence of added antibody colony counts increased 150%–240% during the 60-min incubation [15].

Opsonophagocytosis assay. Enhancement of phagocytosis by antibody and complement was measured by uptake of [^{3}H]thymidine-labeled Hib cells by human polymorphonuclear leukocytes (PMNL) as described [24, 25]. In brief, log-phase radiolabeled Hib cells were resuspended in VBS-BSA (no added Ca or Mg) to a final concentration of ~2×10^9 cfu/ml ($5–7 \times 10^6$ counts per minute (cpm)/ml). The labeled Hib cells were preopsonized with different concentrations of IgG subclass fractions, or subclass-specific affinity-purified anti-PRP antibody, and 25% complement in a final volume of 250 μl. The complement source was serum from a previously described patient with agammaglobulinemia [15] and was the same as that used in the bactericidal assay described above. To minimize complement-mediated bacteriolysis in the opsonic assay, preopsonization was performed in VBS-BSA^{++} supplemented with 0.2% glucose and 5% Bacto Fildes Enrichment (Difco Laboratories, Detroit) [26].

One healthy human donor was used as a source of PMNL. PMNL were separated from the peripheral blood by dextran sedimentation and centrifugation at 300 *g* for 30 min over lymphocyte separation media (Bionetics Laboratory Products, Kensington, MD) [24]. The PMNL in the pellet were resuspended to a concentration of 2×10^7 cells/ml. The phagocytic mixture included PMNL and Hib cells at a ratio of 1:10. The mixture was incubated for 60 min in a shaking water bath at 37°C, and the PMNL-associated bacteria were separated from the free bacteria by differential centrifugation as described [25]. For calculation, uptake of Hib preopsonized with complement and 10% immune serum pool was assumed to yield 100% because it approached the upper limit of uptake of the assay [25]. The undiluted immune pool contained 164 μg/ml anti-PRP antibody. Uptake of Hib preopsonized with 25% agammaglobulinemic serum (complement source) with 10% heat-inactivated agammaglobulinemic serum added instead of immune serum yielded 10%–19% uptake of Hib compared with 100% assigned to Hib opsonized with the 10% immune serum pool and complement and was considered to represent background. After subtraction of the background, the results of the opsonic assay were expressed as 100 times the ratio of uptake of Hib by PMNL to uptake of Hib preopsonized with complement and 10% immune serum pool.

Rat protection assay. Infant rats (8 days old) from seven litters were mixed and randomized to receive 0.1–0.2 ml of different dilutions of antibody or buffer alone (controls) by subcutaneous injection at time 0. The animals were returned to the mothers and

challenged 20 h later with intraperitoneal injection of ~10^2 log-phase cells of Hib strain Eagan. Strain Eagan was chosen for these experiments to allow direct comparison of the results with our previous data [16]. With the inoculum used, 100% of control animals treated in previous studies with buffer alone developed bacteremia at 24 h [16, 27]. As in our previous studies, blood specimens were obtained by intracardiac puncture the day after challenge. Samples of 100 μl, 10 μl, and a dilution corresponding to 1 μl were plated onto chocolate agar. The geometric means of the cfu/100 μl for each treatment group of animals were compared by a *t* test. For calculation of the geometric means, animals with no detectable bacteremia were assigned values of 1 cfu/100 μl.

Results

IgG1 and IgG2 Anti-PRP Antibody Prepared from Pooled IgG

Purity of subclass fractions. Table 1 presents the concentrations of the different IgG subclasses in each of the eight fractions as determined by the capture ELISA for total IgG1, IgG2, and IgG4. IgG3, which represented <2% of original IgG pool, was not measured in the fractions. With one exception, the subclass fractions that were depleted of IgG1 or IgG2 and those that were eluted from the MAb immunoabsorbent columns contained <1% of the "unwanted" subclass. The only exception was affinity-purified IgG2 (fraction IV), which contained 2.3% IgG1.

The IgG1- and IgG2-depleted IgG fractions contained up to 10.9% IgG4 (table 1). However, the IgG affinity-purified anti-PRP antibody had only 4% IgG4. Because of the relatively small contribution of IgG4 antibody to the antibody response to vaccination, the antibody fractions were considered pure.

Bactericidal activity. Table 2 summarizes the bactericidal activity of the IgG1 and IgG2 anti-PRP antibody fractions.

Table 1. IgG subclass composition of the fractions.

Starting material, subclass fraction	% of subclass IgG1	IgG2	IgG4
IgG affinity-purified anti-PRP antibody*			
Affinity-purified IgG1 (II)	99.0	0.8	0.2
Affinity-purified IgG2 (IV)	2.3	96.4	1.3
IgG depleted of IgG2 (III)	94.7	0.8	4.4
IgG depleted of IgG1 (I)	0.2	97.8	2.0
IgG pool†			
Affinity-purified IgG1 (VI)	98.8	0.2	1.0
Affinity-purified IgG2 (VIII)	<0.1	99.9	<0.1
IgG depleted of IgG2 (VII)	88.4	0.7	10.9
IgG depleted of IgG1 (V)	<0.1	99.2	0.8

NOTE. Sum of IgG1, IgG2, and IgG4 was assumed to be 100%. Numbers in parentheses are fractions (defined in figure 1).

* Subclass composition of IgG affinity-purified anti-PRP antibody was 48% IgG1, 48% IgG2, and 4% IgG4 as determined by capture ELISA.

† Subclass composition of IgG pool was 59% IgG1, 38% IgG2, 1% IgG3, and 2% IgG4 as determined by particle concentration fluorescence immunoassay [18].

In the low-inoculum bactericidal assay, about twofold less affinity-purified IgG1 anti-PRP antibody was needed for 50% killing than IgG2 antibody (56 vs. 112 pg/cfu, $P < .01$ by *t* test). Although this difference in activity was small, the results were consistent in multiple assays ($n = 3$). Also, the results assaying IgG affinity-purified anti-PRP depleted of IgG2 or IgG1 (fractions III and I, respectively) were similar to those observed with the respective IgG1 and IgG2 anti-PRP antibodies eluted from the MAb immunoabsorbent columns ($n = 6$ assays, table 2).

To examine the bactericidal activity at a high ratio of bacteria to antibody, we used the high-inoculum assay. The difference in activity found between IgG1 and IgG2 antibody was much greater than that with the low-inoculum assay. With 5 × 10^5 cfu/ml Hib cells, 1 μg/ml affinity-purified IgG1 anti-PRP antibody killed 4.0–4.5 logs of Hib cells, whereas comparable concentrations of IgG2 antibody killed only 0.6–1.2 logs ($P < .001$, table 2). Again, the results were comparable when assaying IgG anti-PRP antibodies depleted of IgG2 or IgG1 or the corresponding IgG1 and IgG2 anti-PRP antibodies eluted from the MAb immunoabsorbent columns (table 2).

These assays used anti-PRP antibody fractions that had undergone one or two elution procedures during their preparation. For example, the anti-PRP antibody fractions depleted of IgG1 or IgG2 had undergone elution during preparation of the IgG affinity-purified anti-PRP antibody that served as the starting material. Thus, one potential explanation for the greater ability of IgG1 than IgG2 anti-PRP antibody to activate complement-mediated lysis of Hib could be a difference in susceptibility of the different IgG subclasses to the denaturing effects of the reagents used to elute the fractions. Therefore, we also measured the bactericidal activity of IgG fractions that were depleted only of IgG1 or IgG2 (fractions V and VII) or had only been eluted from the MAb-absorbent columns (fractions VI and VIII). Although these fractions contained both anti-capsular and anti-noncapsular antibodies as well as normal IgG, the respective bactericidal activities were nearly

Table 2. Bactericidal activity of IgG1 and IgG2 affinity-purified polyclonal anti-PRP antibody.

Fraction, affinity-purified anti-PRP antibody	Bactericidal activity: Anti-PRP (pg/cfu) for 50% killing*	Log_{10} decrease in cfu/ml†
II, affinity-purified IgG1‡	56 ± 1	4.5 ± 0.7
IV, affinity-purified IgG2‡	112 ± 5	1.2 ± 0.5
III, depleted of IgG2	45 ± 4	4.0 ± 0.6
I, depleted of IgG1	115 ± 8	0.6 ± 0.1

NOTE. Fractions are defined in figure 1.

* Low-inoculum assay = 3 × 10^3 cfu/ml of Hib, 20% complement, and serial twofold dilutions of antibody. Data are mean ± SE of three assays for fractions II and IV and six assays for fractions III and I.

† High-inoculum assay = 5 × 10^5 cfu/ml, 20% complement, and 1 μg/ml of anti-PRP antibody. Data are mean ± SE of three assays.

‡ Eluted from anti-IgG1 or anti-IgG2 affinity column.

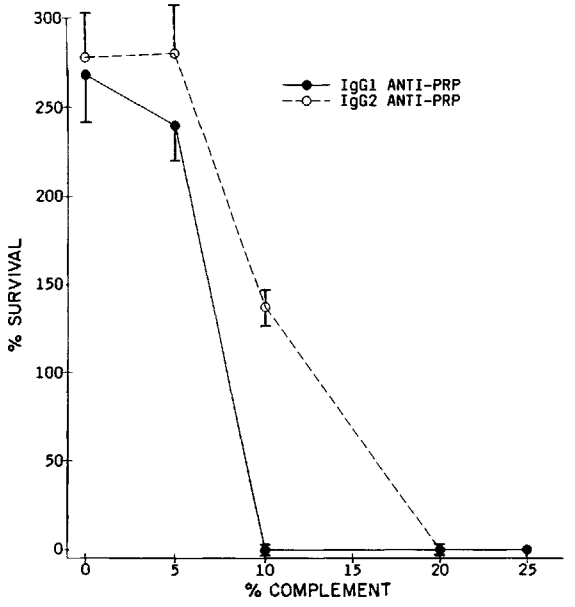

Figure 2. Survival of Hib in different concentrations of complement in presence of 0.5 μg/ml polyclonal IgG1 anti-PRP antibody or 1.0 μg/ml polyclonal IgG2 anti-PRP antibody (fractions III and I, respectively). These antibody concentrations were 2.5-times higher than 50% bactericidal concentrations. Data are mean ± SE of three assays. Hib inoculum concentrations were 2.9–3.9 × 10^3 cfu/ml.

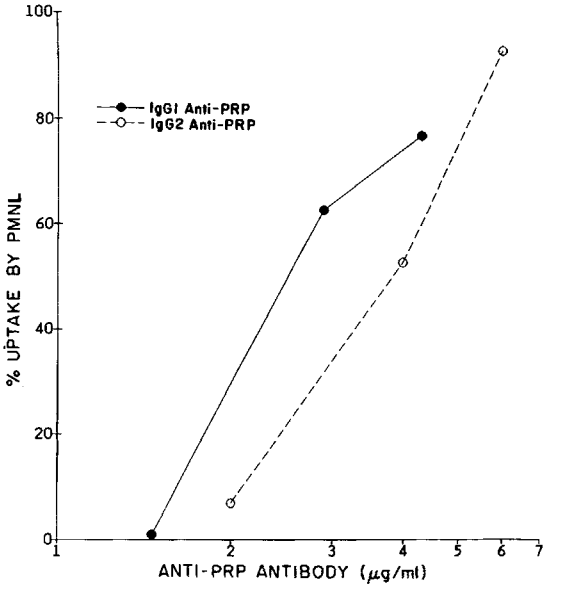

Figure 3. Representative experiment showing effect of preopsonization with complement and polyclonal IgG1 or IgG2 anti-PRP antibody (fractions III and I, respectively) on percentage of uptake of radiolabeled Hib by PMNL. After subtraction of background, results were expressed as 100 times ratio of uptake of Hib to uptake of control Hib preopsonized with complement and 10% immune serum pool, assumed to yield 100% uptake [25].

identical to those obtained with the affinity-purified IgG1 and IgG2 anti-PRP antibody fractions. With the IgG fractions, the anti-PRP antibody concentrations required for 50% killing of Hib cells in the low-inoculum assay were 42 and 36 pg/cfu for IgG1 fractions VI and VII and 145 and 139 pg/cfu for IgG2 fractions V and VIII, respectively (fractions defined in figure 1). Because no bactericidal activity was detected with these IgG fractions after absorption of anti-PRP antibodies, the contribution of anti-noncapsular antibodies to bactericidal activity appears to have been minimal.

Efficiency of bactericidal activity may depend on concentrations of complement. Thus, we examined the concentration of complement sufficient to lyse Hib cells in the presence of IgG1 or IgG2 anti-PRP antibody (figure 2). In this experiment, the low-inoculum bactericidal assay was performed at complement (agammaglobulinemic serum) concentrations of 0, 5%, 10%, 20%, and 25%. To compare the complement concentrations required for bacteriolysis by IgG1 and IgG2 antibody, we used an anti-PRP antibody concentration that was ~2.5-times higher than the 50% bactericidal concentration: 0.5 μg/ml for IgG1 and 1.0 μg/ml for IgG2. The antibodies used in this assay were affinity-purified anti-PRP depleted of IgG1 or IgG2 (fractions I and III). At antibody concentrations of 0.5 and 1.0 μg/ml, respectively, IgG1 anti-PRP required 8.4% ± 0.1% (mean ± SE) complement and IgG2 anti-PRP required 16.5% ± 0.3% complement for 50% killing ($P < .001$). Although this difference is small, the results were consistent in three independent experiments. Also, when the IgG1 and IgG2 anti-PRP antibodies were both tested at 0.5 μg/ml, the difference in the complement concentration required for 50% lysis was magnified (8.4% and 20%, respectively). Thus, IgG1 antibodies require less complement than do IgG2 antibodies for bacteriolysis of Hib.

Opsonic activity. A representative experiment comparing the ability of IgG1 or IgG2 affinity-purified anti-PRP antibody (fractions III and I, respectively) together with complement to enhance the uptake of Hib cells by human PMNL is shown in figure 3. IgG1 antibody appeared to have more opsonic activity than did IgG2 antibody, although the difference was much less than that observed with the bactericidal assays. In three independent assays, the antibody concentration required for 50% of control uptake of Hib cells by PMNL was 0.028 ± 0.002 pg/cfu (mean ± SE) Hib for IgG1 anti-PRP compared with 0.036 ± 0.001 pg/cfu for IgG2 anti-PRP ($P < .01$).

Bactericidal Activities of Clonal IgG1 and IgG2 Antibodies Prepared from Serum of Individual Donors

To examine if V regions influence functional potency of antibodies, we measured bactericidal activity in clonally purified IgG1 and IgG2 anti-PRP antibodies from serum of eight adults immunized with PRP vaccine. The amino acid sequences of the *N* terminus of these antibodies have been determined [12]. In the low-inoculum bactericidal assay, lower concentrations

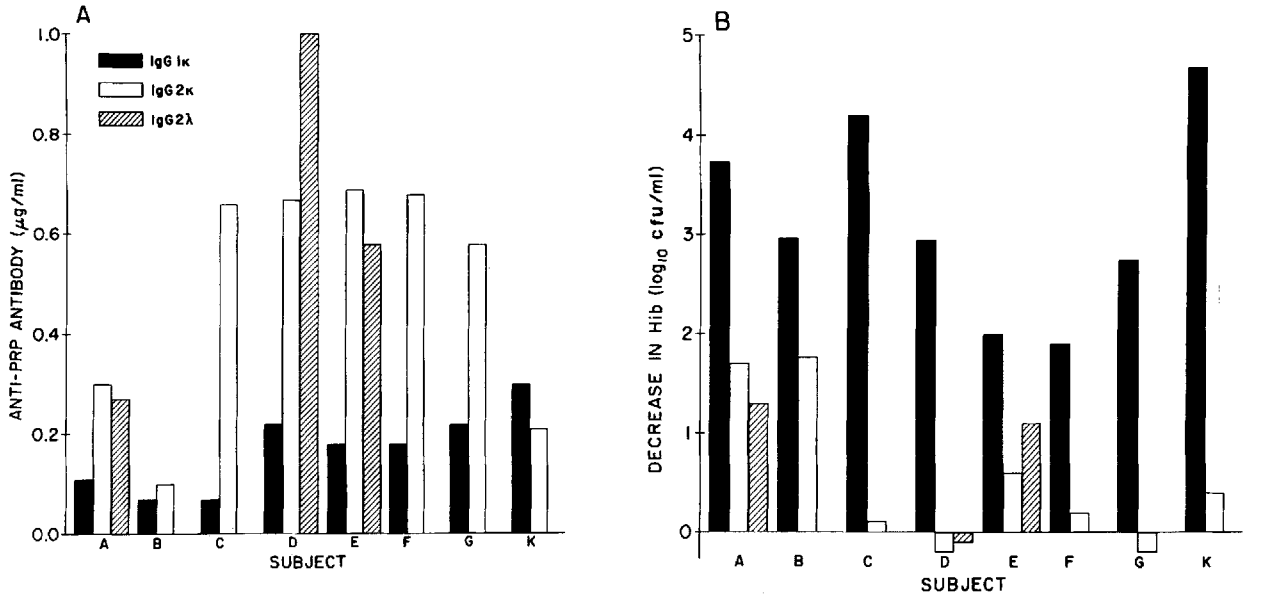

Figure 4. Complement-mediated bactericidal activity of clonal subclass anti-PRP antibody from adults immunized with PRP. Heavy-chain V regions of all seven subjects tested used V_HIII family [12]. Light-chain V regions (V_L) used in five subjects were mainly κII (IgG1 and IgG2 for each of subjects A, B, and C; IgG1 for subjects E and K); κI (IgG2 for subject K); κIII (IgG1 for subject K); and λ (IgG2 for subjects A, D, and E). **A**, Concentration of anti-PRP antibody needed for 50% killing in low-inoculum bactericidal assay (3×10^3 cfu/ml and 20% complement). **B**, Log decrease in Hib cells in high-inoculum bactericidal assay (5×10^5 cfu/ml and 20% complement). Data are means of three assays.

of IgG1 than IgG2 anti-PRP antibody were required to achieve 50% killing for six of eight subjects (figure 4A). In the remaining two subjects (B and K), the bactericidal activity of the respective IgG1 and IgG2 antibodies was not significantly different. In these two subjects, the activity of the IgG2 antibodies was greater than that of the IgG2 antibody of the other subjects. Among the three subjects showing both IgG2λ and IgG2κ responses (subjects A, D, and E), there were no consistent differences in activity of the IgG2 antibodies with either κ or λ light chains. Among the eight subjects, the mean ± SD concentrations of IgG1 and IgG2 antibodies needed for 50% killing of Hib were 51 ± 24 and 152 ± 78 pg/cfu, respectively ($P < .01$). These mean concentrations were similar to those required for 50% killing by the IgG1 and IgG2 polyclonal antibodies prepared from the IgG pool from four other immunized adults (56 and 112 pg/cfu, respectively, table 2).

Figure 4B shows the results of measurement of bactericidal activity of 1 μg/ml of clonally purified IgG1 and IgG2 anti-PRP antibodies from the serum from the same eight individuals using the high-inoculum bactericidal assay. For all eight subjects IgG1 anti-PRP antibody had higher activity than the respective IgG2 anti-PRP antibody, but in two (subjects A and B), the activity of the IgG2 antibodies was comparable to that of the IgG1 antibodies of two other subjects (E and F). In five of the eight subjects (C, D, F, G, and K), high levels of bactericidal activity were detected at 1 μg/ml IgG1 antibody, but there was no significant bactericidal activity detected in the corresponding IgG2 antibodies when tested at the same antibody concentration.

Rat protective activity. To determine whether the differences in the relative bactericidal or opsonic activity of IgG1 and IgG2 antibody reflect differences in in vivo protection, passive protection experiments were performed in infant rats. For these experiments, a new serum pool was prepared from postvaccination sera of seven additional adults immunized with PRP. These adults were selected on the basis of high serum antibody response (27–262 μg/ml) and the presence of both IgG1 and IgG2 anti-PRP antibody. Affinity-purified anti-PRP antibody was prepared from the serum pool and separated into IgG1 and IgG2 fractions using MAb immunosorbent columns as described above. However, for purification of IgG2, MAb HP6014 was used instead of HP6016. In addition, the eluted IgG1 and IgG2 fractions were passed over the "wrong" columns to remove residual contaminating IgG2 or IgG1, respectively. Both fractions contained <1% IgG4 as determined by the particle concentration fluorescence immunoassay [18]. The IgG2 fraction contained <1% contamination by IgG1 as determined by the IgG subclass capture ELISA. The IgG1 fraction contained <1% IgG2 when measured with MAb HP6014. The IgG1 and IgG2 fractions were assayed for anti-PRP antibody by RABA and tested for protective activity in infant rats challenged with ~10^2 cfu of Hib strain Eagan injected intraperitoneally.

Figure 5 shows the geometric mean ± SE of the cfu/100

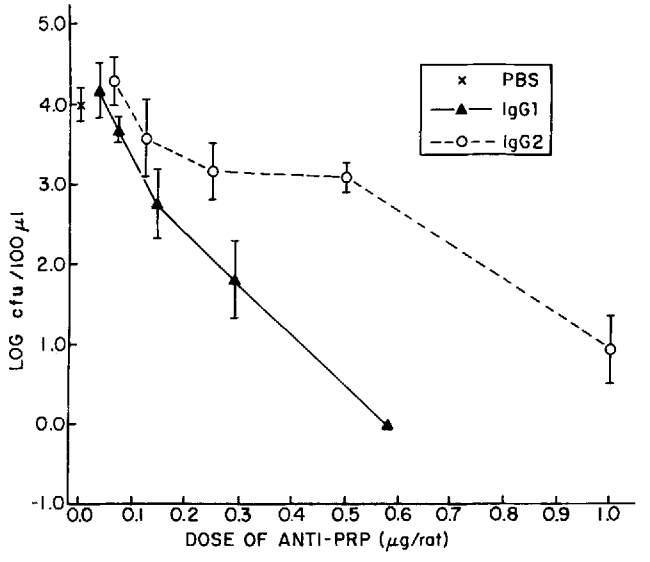

Figure 5. Effect of pretreatment with different amounts of IgG1 or IgG2 anti-PRP antibody on level of Hib bacteremia 24 h after challenge of infant rats with ~10^2 cfu of Hib injected intraperitoneally. Data are mean $\log_{10} \pm$ SE of cfu/100 µl of blood. Each treatment group consisted of five animals except for controls treated with buffer alone ($n = 8$). Animals pretreated with 0.3 µg of IgG1 anti-PRP had significantly lower geometric mean cfu/100 µl than animals pretreated with 0.5 µg of IgG2 antibody ($P < .03$). For analysis animals with negative cultures were assigned values of 1 cfu/100 µl.

µl of blood of groups of rats given different amounts of IgG1 or IgG2 antibody in PBS containing 2% BSA or buffer alone (PBS-albumin). Significant protection was observed in animals given 0.14–0.25 µg of IgG1 or IgG2 anti-PRP antibody compared with the controls ($P < .05$), but the difference between the geometric means of the cfu/100 µl of the two treatment groups at this dosage range was not significant. However, pretreatment with 0.3 µg of IgG1 antibody per rat resulted in a lower level of bacteremia (geometric mean, 90 cfu/100 µl) compared to pretreatment with 0.5 µg of IgG2 antibody per rat (geometric mean, 681 cfu/100 µl; $P < .03$). Further, at 0.6 µg of IgG1 antibody per rat, none of the animals developed detectable bacteremia, whereas three of the five animals pretreated with 1 µg of IgG2 antibody had detectable bacteremia (10, 50, and 100 cfu/100 µl).

Discussion

We evaluated the bactericidal and opsonic activity of IgG1 and IgG2 antibodies to PRP. The bactericidal activity of IgG1 anti-PRP antibody was more efficient than IgG2 antibody in several experiments. When it was measured in the low-inoculum bactericidal assay, a twofold difference was found. The ratio of Hib cells to antibodies in this assay is about in the range that one might expect in the blood of infected children. In the high-inoculum bactericidal assay, where Hib cells interact with limiting dilutions of antibody and complement, the difference in the bactericidal activity between IgG1 and IgG2 was >3 logs. The conditions of this high-inoculum assay are probably not representative of those in the bloodstream of a child with infection, but may be encountered in infected body fluids, such as cerebrospinal fluid [28, 29]. The antibody concentrations used in this study were calibrated by two antigen-binding assays: RABA and IgG ELISA. The results of these assays were in excellent agreement. For example, 19 clonally purified IgG1 or IgG2 anti-PRP antibodies were quantified by both assays. The respective mean concentrations were 36.9 and 39.0 µg/ml, and the paired values from the 19 samples correlated at $r = .97$. Therefore, it is unlikely that the differences in the bactericidal activity between the IgG1 and IgG2 antibodies were a result of assay variability.

The present study greatly expands an earlier one that investigated complement-dependent bactericidal activity of IgG1 and IgG2 anti-PRP antibody using a low inoculum of Hib cells of a different strain (Eagan) [16]. In that study, the concentration of anti-PRP antibody required for 50% killing was 0.21 µg/ml for IgG1 and was 0.42 µg/ml for IgG2. The conclusion was that there was no significant difference between the activity of the two subclasses. However, a consistent twofold difference also was found with the low-inoculum assay in the present study (i.e., 0.15 and 0.39 µg/ml of IgG1 and IgG2 antibody, respectively). The similarity of the results from the two studies despite the use of different Hib strains, different antibody pools, and different fractions of affinity-purified anti-PRP antibody suggest that this twofold difference is probably real. Further, the present results using the high-inoculum assay suggest that at limiting concentrations of antibody and complement the difference between the functional activity of IgG1 and IgG2 anti-PRP antibody may be considerable.

The finding of a difference in the bactericidal potency between IgG1 and IgG2 antibodies is consistent with previous reports of studies of complement activation. Using red blood cells sensitized with immunoglobulin, Ishizaka et al. [30] showed that IgG1 activated the complement cascade to create the membrane attack complex better than did IgG2 antibody. Müller-Eberhard [31] found that IgG1 reacted better with C1q than did IgG2. We also found that a lower concentration of complement was capable of lysing Hib cells sensitized with IgG1 than with IgG2 anti-PRP antibody (figure 2). The more efficient activation of the complement system by IgG1 anti-PRP antibody than by IgG2 antibody may explain, in part, its greater bactericidal activity.

We found small but consistent differences in the opsonic activity of IgG1 and IgG2 anti-PRP antibody in an assay that used PMNL as phagocytes and a high inoculum of Hib (figure 3). Because opsonic activity may be more important than bacteriolysis in conferring protection against invasive Hib dis-

ease, the opsonic results suggest that IgG1 antibody is slightly superior to IgG2 anti-PRP antibody in conferring protection against Hib disease. The in vivo rat protection data were consistent with the bactericidal and opsonic results and indicated that IgG1 antibody has about two- to threefold greater protective activity than IgG2 antibody (figure 5). The biologic relevance of this difference is unknown, but even a twofold difference might be important in a child with low concentrations of serum anti-PRP antibody.

Finally, the results of the bactericidal assays using clonally purified antibodies from individual sera confirm the results obtained with purified IgG1 and IgG2 pooled polyclonal anti-PRP antibodies, that is, that lower concentrations of IgG1 antibody are capable of activating complement-mediated killing of Hib than IgG2 antibody. However, it is of interest that clonally pure anti-PRP IgG2 antibodies of certain subjects (e.g., A and B) appeared to have similar activity to the IgG1 antibodies of other subjects (e.g., E and F, figure 4B). At present, we cannot explain these differences. There was no correlation between the levels of bactericidal activity and classification of these clonal antibodies with respect to V_H or V_L gene families [12] or antigenic fine specificity as determined by cross-reactivity with the *Escherichia coli* K100 polysaccharide [10]. It is possible that differences in antibody avidity may contribute to the differences in individual antibody function [24, 32], but there were insufficient quantities of the clonally purified antibodies to test avidity.

In conclusion, IgG1 anti-PRP antibody was shown to have better bactericidal, opsonic, and rat protective activity than IgG2 antibody in several different assays. However, because in most instances the differences were small, it is likely that both IgG1 and IgG2 anti-PRP antibodies are effective in conferring protection against Hib disease.

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