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Complement Activation by Polyclonal Immunoglobulin G1 and G2 Antibodies against *Staphylococcus aureus*, *Haemophilus influenzae* Type b, and Tetanus Toxoid

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To obtain information on effector functions of human immunoglobulin G2 (IgG2), we have measured the complement-activating properties of polyclonal IgG subclass antibodies against bacterial antigens. IgG1 and IgG2 were purified from serum samples from five healthy individuals, and complement activation was measured with different bacterial antigens. We used *Staphylococcus aureus* Wood 46 (STAW), which is a common antigen, *Haemophilus influenzae* type b (Hib), which is a common pathogenic microorganism in children, and formaldehyde-inactivated tetanus toxin (TT). Bacteria were incubated with antibodies and then incubated with sera from agammaglobulinemic patients as a complement source, and C3c deposition was measured by enzyme-linked immunosorbent assay. We found that anti-STAW IgG2 activated complement to a level similar to that of anti-STAW IgG1. Anti-Hib IgG1 complement activation was as much as seven times higher than that of anti-Hib IgG2 in four individuals. In one individual, anti-Hib IgG2 was more effective in complement activation than anti-Hib IgG1. Anti-TT antibodies showed patterns similar to those of anti-Hib. Our results indicate that IgG2 antibodies may contribute significantly to antibacterial defense. Also, individual differences in antibody effector functions should be taken into account when evaluating the immune status of patients and during early phase 1 studies of new vaccines.

Decreased concentrations of immunoglobulin G2 (IgG2) are often associated with recurrent bacterial infections (30, 35, 37). A causal relationship is not clear, as IgG2 antibodies are considered less effective in mediating complement activation than IgG1 antibodies, and their binding to Fcγ (constant fragment of IgG) receptors is supposed to be weaker than that of IgG1 (6).

Much of the knowledge of effector functions of IgG subclasses has been obtained from studies with aggregated myeloma proteins, showing that IgG2 binds complement less effectively than IgG1 (23, 34). More recently, chimeric monoclonal antibodies (MAbs) with identical variable regions but different constant regions of human origin were used to study Fc-mediated effector functions. Again, IgG1 proved more effective than IgG2 in mediating binding of the first complement component (C1q), C4 activation, and complement-mediated cytolysis (5, 8, 14, 27). However, these findings may not reflect the physiologic activity of polyclonal human antibodies interacting with common bacterial antigens in vivo. In other studies, polyclonal antibodies from a pool of hyperimmune sera were used, thus masking differences between the donors (15, 43). Recently, Amir et al. (2) found that in pooled sera, affinity-purified IgG1 against the capsular polysaccharide (polyribosyl ribitol phosphate [PRP]) of *Haemophilus influenzae* type b (Hib) was more active than anti-PRP IgG2 in several test systems (bactericidal, opsonization, and rat protection assays). However, in sera from individual donors vaccinated with PRP vaccine, anti-PRP IgG2 preparations from two individuals were similar to the anti-PRP IgG1 preparations from two other individuals.

Differences in the functional affinities of antibodies may influence these analyses, since correlations between antibody affinity and effector functions have been observed (1, 17, 20).

In the present study, we have investigated whether IgG2 antibodies have complement activation capacity, which would enlighten IgG2 deficiency-related disease. We have chosen a strategy that would reflect the physiologic situation as much as possible, by using polyclonal IgG subclass antibodies and common bacterial antigens. Human IgG1 and IgG2 antibodies from five individuals were purified by affinity chromatography with Sepharose-protein A. Different G2m(n) allotypes were included for the investigation of differences in complement-activating properties. The G2m(n) allotype substitution in the CH_2 domain of the IgG molecule has not yet been located precisely, but it may be close to the binding and activation site of C1q (6, 38), the first component of the complement cascade, and might thus influence complement-activating properties of IgG2. Complement-activating properties of antibodies against *Staphylococcus aureus* Wood 46 (STAW), Hib, and formaldehyde-inactivated tetanus toxin (TT) were analyzed. We used a commensal nonencapsulated gram-positive microorganism, *S. aureus*, which is a common antigen, and against which most people should have protective antibodies; an encapsulated gram-negative microorganism, Hib representative for the invasive disease isolates in Europe (39); and TT, as a protein and reference antigen, with which most individuals have been immunized and have protective IgG1 and IgG2 antibodies. C3c deposition on the bacterial surface was measured by enzyme-linked immunosorbent assay (ELISA), using poly-

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clonal anti-C3c, which recognizes native C3 and C3b, including C3bi. We show that anti-STAW IgG2 and IgG1 activated complement almost equally well. Anti-Hib IgG2 and anti-TT IgG2 showed interindividual differences: some IgG2 preparations showed slightly more complement activation than IgG1 preparations, but most were less effective than IgG1. Our results indicate that IgG2 antibodies may have an important role in defense against these bacteria.

MATERIALS AND METHODS

Materials. Sephacryl S-300 and protein A-Sepharose CL-4B were from Pharmacia, Uppsala, Sweden. The Amicon concentrator (cell model M-3) and Diaflo ultrafiltration membranes (YM10) were from Amicon, Danvers, Mass. Mouse MAbs specific for IgG subclasses (MH 161-1-MO1, MH 162-1-MO2, MH 163-1-MO2, and MH 164-4-MO2), horseradish peroxidase (HRP)-conjugated murine MAbs specific for IgG subclasses (MH 161-1-ME2, MH 162-1-ME2, MH 163-1-ME3, and MH 164-4-ME3) and specific for human IgG (MH 16-1-ME) were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. The specificity of these antibodies has been documented extensively (24, 25, 29, 33). Polyclonal rabbit anti-IgA (KH 14-22-P), anti-IgM (KH 15-24-P), and anti-IgG (KH 16-109-P) antibodies were from the same institute. Human IgG1 (clone 151) specific for TT was a gift from R. F. Tiebout, CLB. Rabbit anti-human C3c (code no. A062), HRP-conjugated rabbit anti-human C3c (code no. P213), HRP-conjugated rabbit anti-mouse IgG (code no. P260), and HRP-conjugated rabbit anti-human IgG (code no. P214) were purchased from Dako, Glostrup, Denmark. Mouse monoclonal anti-human SC5b-9 (neoantigen; code no. A239) was obtained from Sanbio bv, Uden, The Netherlands.

TT and purified PRP were obtained from the National Institute for Health, Environment and Toxicology (RIVM; Bilthoven, The Netherlands). We used a representative encapsulated Hib, strain 760705, which causes the majority of invasive Hib disease in Europe (39). Hib and unencapsulated protein A-deficient STAW (270581) bacteria were kindly provided by L. van Alphen (Department of Microbiology, University of Amsterdam, Amsterdam, The Netherlands). Hib was cultured in brain heart infusion broth containing hematin and NAD^+, and STAW was cultured in nutrient broth 2. Bacteria were harvested in log phase, washed three times with phosphate-buffered saline (PBS) (140 mM NaCl, 9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4; pH 7.4), and resuspended in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Phosphate buffer containing Ca^{2+} and Mg^{2+} (PiCM buffer) (pH 7.2 to 7.4) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $MgCl_2$, 0.6 mM $CaCl_2$, 1% (wt/vol) glucose (all from Merck, Schuchardt, Hohenbrunn, Germany), and 2.5% (vol/vol) human serum albumin (from CLB, Amsterdam, The Netherlands). Tween 20, $NaHCO_3$, citrate, and Na_2HPO_4 were also from Merck. Tetramethyl-benzidine was purchased from Sigma, St. Louis, Mo. Flat-bottom, 96-well microtiter plates (Immunolon M129A) were from Greiner, Kloten, Switzerland.

Serum samples were obtained from healthy laboratory personnel, and samples with large amounts of anti-Hib and anti-Sta IgG1 and IgG2 were selected. Individuals with different IgG2 G2m(n) allotypes were chosen. Sera from patients with agammaglobulinemia (and with normal hemolytic complement activity) were used as source of complement. All agammaglobulinemic serum samples were stored at −80°C and thawed at 4°C just before use.

Purification of IgG subclasses. Serum samples were obtained from five healthy adults. Complement was heat inactivated (45 min at 56°C). IgG was separated from other serum proteins by gel filtration at 4°C, using Sephacryl S-300; PBS was used as the elution buffer.

IgG-, IgM-, and IgA-rich fractions were pooled. The IgG antibodies were applied to a protein A-Sepharose column (20 by 1.1 cm; 21-ml bed volume) at 4°C by the method of Duhamel et al. (9). IgG was eluted with 0.02 M citrate brought to pH 5.0 with Na_2HPO_4 (approximately 0.04 M Na_2HPO_4) and then with a solution with a pH gradient from pH 5.0 to 3.0 (0.02 M citrate brought to pH 3.0 with Na_2HPO_4 [approximately 0.008 M Na_2HPO_4]). Fractions were collected in tubes that contained 0.5-ml portions of 0.25 M Na_2HPO_4 (pH 8.9) to neutralize the acid pH of the fractions immediately. IgG subclass content was measured (see below), and IgG1-, IgG2-, and IgG3-rich fractions from each individual were pooled. We purified IgG4 from one individual by immunoabsorption using Sepharose anti-IgG4 (an anti-IgG4 MAb, MH 164-4-MO2), essentially as described by Nagelkerken et al. (28). The IgG subclass preparations were concentrated with an Amicon concentrator and a YM10 membrane, diluted in PiCM buffer, and stored in aliquots at −80°C.

IgG subclass assay. IgG subclasses were measured by noncompetitive two-site ELISAs (29). Briefly, mouse MAbs specific for IgG subclasses (MH 161-1-MO1, MH 162-1-MO2, MH 163-1-MO2, and MH 164-4-MO2) were used to coat microtiter plates. Uncoated sites were blocked, and dilutions of fractions or subclass preparations were added to the wells and incubated (2 h at room temperature). The Dutch reference serum H00-03 (CLB) was used as a standard. Bound IgG antibody subclasses were detected by HRP-conjugated murine anti-human IgG MAb (MH 16-1-ME). The results of IgG subclass assays by the ELISAs were the same as those by radial immunodiffusion assay (29).

IgG2 G2m(n) allotyping. The G2m(n) allotypes of the five donors were determined by double immunodiffusion assay by the method of Rautonen et al. (32). Three of the selected donors were homozygous G2m(n) negative (n^-/n^-), one was homozygous G2m(n) positive (n^+/n^+), and one was heterozygous (n^+/n^-).

Complement deposition assay. Deposition of complement C3c on bacteria was measured by ELISA, essentially as described earlier (16). Preparation and coating of STAW, Hib, and TT were performed as previously described for ELISAs of antibodies to bacterial antigens (33). STAW and Hib were coated (150 μl per well; 2 h at room temperature) at concentrations of approximately 5×10^6 and 1×10^7 CFU per ml, respectively, and TT in a concentration of 1.5 Lf/ml. TT was coated at least 48 h before the assay. Free binding sites were blocked with 150 μl of PiCM buffer. Plates were washed first with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween) and then washed three times with PBS. At least four serial dilutions of heat-inactivated samples made in PiCM buffer were applied (100 μl per well) and incubated for 1 h at 37°C. Standard and control sera were applied in the same way. Plates were washed again. For a source of complement, we used serum obtained from an agammaglobulinemic patient; a 100-μl portion of serum 1% (vol/vol) in PiCM buffer was added to each well and incubated (30 min, 37°C). After the wells were washed, 100 μl of HRP-conjugated rabbit anti-human C3c (4 μg/ml), diluted in PBS containing 0.1% (wt/vol) gelatin and 0.02% (vol/vol) Tween

20, pH 7.3, was added to each well and incubated (2 h, 37°C). Plates were washed, and 100 μl of substrate was added to each well: 0.11 M acetic acid, 0.01% (wt/vol) tetramethylbenzidine, 1% (vol/vol) dimethyl sulfoxide, and 0.003% (vol/vol) H_2O_2, pH 5.4. The reaction was stopped by adding 100 μl of 2 M H_2SO_4. The anti-C3c reagent recognizes the C3c part of native C3 and C3b, including C3bi (44). Complement activation was expressed either in A_{450}, measured with a Titertek Multiscan MC (Flow Laboratories, Irvine, UK), or in percentage of normal human serum. Complement activation was also expressed per amount of antibacterial antibody bound to the antigen and expressed as a ratio (see below [Table 3]). To do so, antibacterial antibodies were measured by ELISA (33) and expressed in micrograms of bound antibody per milliliter (see next section), and complement activation in the C3c deposition ELISA, was expressed as percentage of normal human serum, measured in an A_{450} range of 0.5 to 1.0. For each antigen, a different serum sample was used as the standard; therefore, the relative complement activation ratios for one antigen cannot be compared with those of another antigen. Normal human sera were selected as standards by using high titers of antibacterial IgG and titration curves relatively parallel to those of the subclass fractions as criteria. The amount of C3c deposition was quantitated as indicated below.

To obtain an independent measure of the amount of C3c that was deposited onto the immune complexes, we performed a two-site ELISA for C3c. Serial dilutions of a standard serum with known levels of C3c were added to the anti-C3c serum-coated wells (2 μg/ml), and the further procedure was performed as described previously (40). The same amounts of HRP-conjugated anti-C3c as in the C3c deposition ELISA were added. Incubation times and enzyme reaction times were equal in both ELISAs. In this way, we were able to compare the A_{450} in the C3c deposition ELISA with the A_{450} in the C3c capture ELISA, in which known amounts of C3c were added. To demonstrate further complement activation, we measured the formation of the terminal complement complex (22) in a manner similar to that of the C3c deposition assay. In this study, we used mouse anti-human SC5b-9 (166 ng/ml) and HRP-conjugated rabbit anti-mouse IgG (0.5 μg/ml).

The intraassay coefficient of variation (CV) of the C3c deposition ELISA was less than 10%. The interassay CV was 25%. Therefore, to compare IgG1 and IgG2 antibody activities, the fractions were applied to one microtiter plate, and the assays were repeated for confirmation of results.

Determination of antibodies. IgG subclass antibodies against STAW, Hib, and TT were measured by ELISA (33). Briefly, whole bacteria and TT were coated in the same way as described above for the C3c deposition ELISA. After the free sites were blocked, samples were added to each well. Antibacterial IgG subclass antibodies in sera were detected by HRP-conjugated MAbs to each of the subclasses (MH 161-1-ME2, MH 162-1-ME2, MH 163-1-ME3, and MH 164-4-ME3). Anti-TT IgG1 (clone 151) was used to calibrate the reference sera (33). Enzyme reaction times of the IgG subclass ELISAs were standardized. Antibodies in the purified IgG subclass fractions were analyzed in the same way, and parallel ELISAs in which the total amount of IgG antibodies bound were detected with HRP-conjugated anti-IgG (P214; Dako) were run. The reproducibility and CVs of these assays was as follows: intraassay CV, <4%; interassay CV, <9%; but 31% for anti-STAW IgG2 (33). The summed amounts of IgG1, IgG2, IgG3, and IgG4 antibodies did not always match with total IgG antibodies. We assume, however, that up-to-twofold differences are still within acceptable limits. Hib capsular polysaccharide PRP was tyraminated as described previously (3), and antibodies against PRP were measured in the same way as in the whole-cell anti-Hib ELISA, after the tyrosylated plates were coated with PRP (5 μg/ml). Reference serum with known amounts of anti-PRP antibodies was used as standard (42) (provided by L. van Alphen). In this study, antibody amounts in microgram equivalents (μgeq) are shortly indicated as micrograms hereafter.

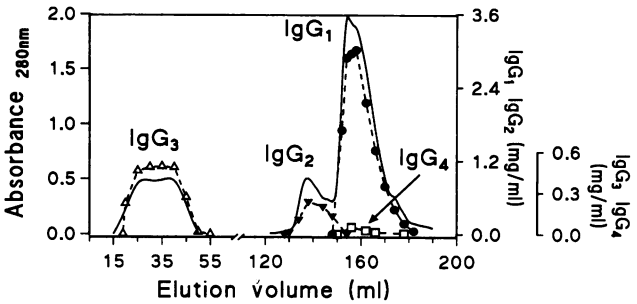

FIG. 1. IgG subclass purification with Sepharose-protein A. IgG (approximately 60 mg of protein) obtained after Sephacryl S-300 gel filtration was loaded onto the column at pH 5.0. After most of the unbound protein (55-ml elution volume) had run through the column, the pH gradient from 5.0 to 3.0 was started. The solid line shows A_{280}. IgG1, IgG2, IgG3, and IgG4 were measured by ELISA.

Statistics. Differences between the ratios of C3c deposition per microgram of antibodies of IgG1 and IgG2 preparations (see Table 3) were tested by the Wilcoxon matched-pair signed-rank test and Student's *t* test.

RESULTS

Purification of IgG1 and IgG2 antibodies. IgG1 and IgG2 antibodies from five individuals were purified. From each individual the IgG-rich fractions, obtained after gel filtration, were loaded onto the Sepharose-protein A column at pH 5.0. A representative elution pattern is shown in Fig. 1. Gradient elution was started after the peak of unbound protein (IgG3) had eluted. The first peak was IgG2, whereas IgG1 eluted from the column approximately 0.5 pH unit later. After gel filtration, the mean recoveries of IgG1 and IgG2 were 80 (range, 64 to 100%) and 84% (65 to 100%), respectively. After affinity chromatography and concentration of antibodies, the mean recoveries were 52% (38 to 65%) for IgG1 and 48% (42 to 63%) for IgG2. Most of the IgG3 was lost in the IgA-rich pool obtained after gel filtration, and the mean recovery of IgG3 was only 28%. IgG1-rich fractions contained 4 to 10% IgG2, and IgG2-rich fractions contained less than 5% IgG1, except for 12% in one preparation. IgG4 was found mainly in the IgG1-rich fractions (1 to 8%).

Specific antibodies. The values for recovery and purity of antibacterial antibodies, measured with anti-IgG subclass monoclonal antibodies, were similar to those for total IgG subclass proteins. Table 1 shows the results of the purification of IgG subclass anti-Hib antibodies for each of the five serum samples. In the IgG1 pools, anti-Hib IgG1 ranged from 87 to 95% of the total anti-Hib. In the IgG2 pools, anti-Hib IgG2 was 84 to 100% of the total anti-Hib. We also measured the anti-Hib antibodies with polyclonal HRP-conjugated anti-IgG. The anti-Hib IgG thus measured (Table 1) corresponded to the sum of the subclass anti-Hib, with

TABLE 1. IgG subclass antibodies against Hib

Source	Antibody[a]	Data on antibodies from the following individual:									
		A		B		C		D		E	
		Concn (μg/ml)	%[b]	Concn (μg/ml)	%	Concn (μg/ml)	%	Concn (μg/ml)	%	Concn (μg/ml)	%
Serum	IgG1 aHib	4.10	23	25.90	58	10.50	80	21.20	79	4.20	52
	IgG2 aHib	12.90	73	18.20	41	2.56	20	5.76	21	3.84	48
	IgG3 aHib	0.40	2	0.20	0	0.02	0	0.00	0	0.00	0
	IgG4 aHib	0.30	2	0.50	1	0.02	0	0.01	0	0.01	0
	IgG aHib	15.2		40.3		13.7		21.0		13.8	
IgG1 fraction	IgG1 aHib	1.60	87	12.60	93	4.20	94	6.10	95	2.10	91
	IgG2 aHib	0.20	11	0.96	7	0.26	6	0.35	5	0.20	9
	IgG4 aHib	0.04	2	0.04	0	0.01	0	0.00	0	0.02	1
	IgG aHib	3.7		14.7		5.3		5.5		4.0	
	IgG aPRP	2.0		4.0		0.7		1.0		6.0	
IgG2 fraction	IgG1 aHib	0.90	16	0.67	7	0.05	9	0.01	1	0.00	0
	IgG2 aHib	4.60	84	8.32	93	0.48	91	0.80	99	1.76	100
	IgG4 aHib	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0
	IgG aHib	6.0		10.0		1.0		1.3		2.3	
	IgG aPRP	12.0		5.0		0.7		1.0		1.0	

[a] IgG1 aHib, IgG2 aHib, IgG3 aHib, and IgG4 aHib, Anti-Hib detected with HRP-conjugated anti-IgG subclass MAbs (see text). IgG aHib, Anti-Hib detected with HRP-conjugated polyclonal anti-IgG (see text). aPRP, Anti-PRP detected with HRP-conjugated polyclonal anti-IgG (see text).
[b] Percentage of total anti-Hib IgG in the sample.

minor discrepancies for the serum from individual E, the IgG1 fraction from individual A, and the IgG2 fraction from individual C. The summary of the purification of anti-STAW, anti-Hib, and anti-TT antibodies from five serum samples is given in Table 2. In the IgG1 fractions, the IgG1 antibodies were 85 92, and 96% of total anti-STAW, anti-Hib, and anti-TT, respectively. In the IgG2 fractions, the IgG2 antibodies were 93, 93, and 94% of total anti-STAW, anti-Hib, and anti-TT, respectively. The antibacterial IgG3 antibody concentrations were very low, and most IgG3 proteins were lost in the IgA-rich fractions after gel filtration, which resulted in the lack of measurable IgG3 antibodies in the subclass fractions. Antibacterial IgG4 antibodies were detected only in serum from one individual (A). The IgG1 preparation from this individual contained some anti-STAW IgG4 (0.15 μg/ml). Other IgG1 (and IgG2) preparations contained less than 2% antibacterial IgG4. Again, antibody concentrations measured with polyclonal HRP-conjugated anti-IgG corresponded to the summed total of the IgG subclass antibodies. We considered the purity to be sufficient to assess the effector functions of IgG1 and IgG2 antibodies separately.

Complement activation assay. Figure 2 shows the C3c deposition with Hib as coated antigen. Higher absorbance signals were obtained when more antigen was coated onto the plates (Fig. 2a) and when higher concentrations of antibodies or complement source were used (Fig. 2b). For further experiments, we used 5×10^6 and 1×10^7 CFU/ml, for STAW and Hib, respectively, and 1.5 Lf/ml for TT for coating the plate, which are the same conditions as in the antibacterial antibody ELISA. Different agammaglobulinemic sera serving as complement source (and with normal hemolytic activity) were compared. An agammaglobulinemic serum preparation with low background levels (no antibodies added) was selected and used for further experiments. For the analysis of antibacterial antibody-dependent C3c deposition, the optimal complement concentration was obtained when 1 μl of agammaglobulinemic serum in 100 μl of PiCM buffer (1% [vol/vol]) per well was used.

Information on the amount of C3c deposited was obtained by performing a C3c capture ELISA at the same time as the C3c deposition assay. A_{450}s of 0.1, 0.2, 0.3, 0.5, 0.7, 0.9, and 1.1 were obtained when 100-μl portions of a solution containing 22, 46, 63, 103, 148, 188, and 228 nM C3c, respectively, were added in the C3c capture ELISA.

Complement activation by IgG1 and IgG2. The IgG subclass preparations from the five individuals were analyzed. When comparing IgG1 and IgG2, we always used the same agammaglobulinemic serum preparation. To assess the relative complement activation properties of the IgG subclass preparations, we applied the amounts of IgG subclass antibodies that had shown similar amounts of antibody bound to the antigen, as determined in the antibacterial antibody ELISAs. Figure 3a shows the results of anti-STAW IgG1 and IgG2 antibodies from individual A. Bound anti-STAW IgG1 and IgG2 antibodies showed almost equal complement activation. The same was found for the antibodies from other individuals, except anti-STAW IgG1 from individual C was more than twice as active as his anti-STAW IgG2 (see Table 3). We calculated that there was no loss of biological activity during the purification process by demonstrating that the biological activities of the purified IgG subclass fractions were the same as those of serum preparations and IgG pool after Sephacryl S-300.

Figure 3b shows complement activation by anti-Hib IgG1 and IgG2 antibodies for two individuals. Anti-Hib IgG1 from individual A was more active than his anti-Hib IgG2 (see also Table 3). Similarly, three other IgG1 preparations were

TABLE 2. Summary of antibody results from five individuals after purification of IgG subclass antibodies against bacterial antigens

Source	Antibody	Anti-STAW		Anti-Hib		Anti-TT	
		Concn[a] (μg/ml)	%[b]	Concn (μg/ml)	%	Concn (μg/ml)	%
Serum	IgG1[c]	0.73	34	13.2	58	3.06	87
	IgG2	1.91	63	8.7	40	0.30	13
	IgG3	0.02	1	0.1	1	0.00	0
	IgG4	0.05	2	0.2	1	0.00	0
	IgG[d]	2.78		20.8		3.3	
IgG1 fraction	IgG1	0.60	85	5.3	92	1.41	96
	IgG2	0.07	12	0.4	8	0.03	4
	IgG4	0.04	3	0.0	1	0.00	0
	IgG	0.85		6.6		1.5	
	aPRP[e]			2.7			
IgG2 fraction	IgG1	0.02	6	0.3	7	0.01	6
	IgG2	0.64	93	3.2	93	0.08	94
	IgG4	0.00	0	0.0	0	0.00	0
	IgG	0.86		4.1		0.3	
	aPRP			3.9			

[a] Mean of concentrations from five individuals (A to E).
[b] Percentage of total anti-STAW, anti-Hib, or anti-TT IgG in the sample.
[c] Antibodies detected with HRP-conjugated monoclonal anti-IgG subclass antibodies (see text).
[d] Antibodies detected with HRP-conjugated polyclonal anti-IgG (see text).
[e] Antibodies against PRP measured with HRP-conjugated polyclonal anti-IgG (see text).

slightly more active than IgG2 from the same person. However, anti-Hib IgG2 from individual E had higher activity than anti-Hib IgG1 (Fig. 3b). Table 3 summarizes the results of complement activation by anti-Hib antibodies for the five individuals.

Since both anti-outer membrane protein antibodies and anti-capsular polysaccharide (PRP) antibodies play a role in the immune response to Hib (19, 21), we measured anti-PRP (see Table 1) in addition to antibodies against whole bacteria. This enabled us to express the results for C3c deposition on whole Hib bacteria relative to the anti-PRP antibodies in the IgG subclass fractions (Table 3). Also, for C3c deposition

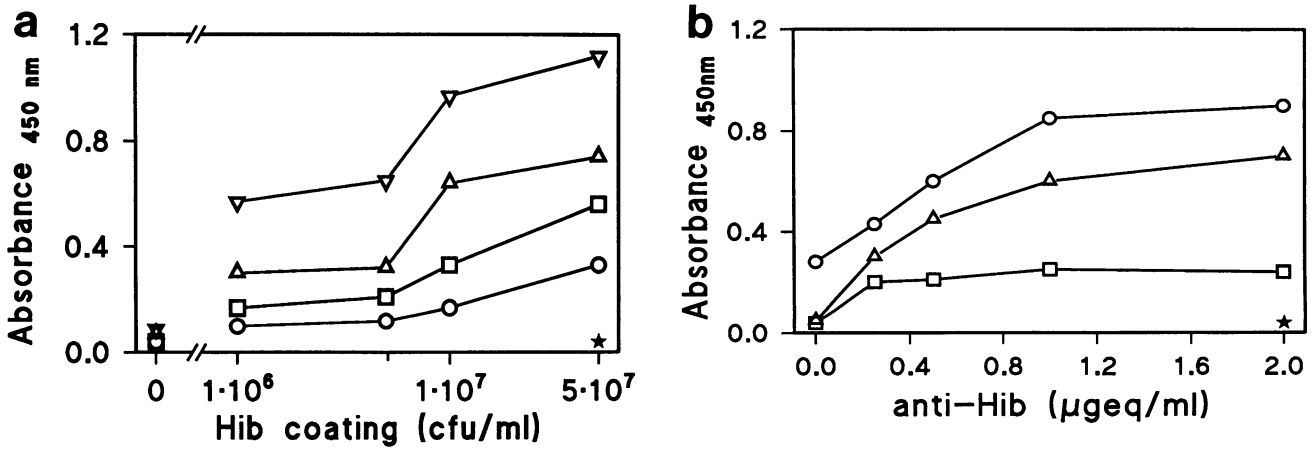

FIG. 2. C3c deposition assay. (a) Titration of Hib coating and antibodies. Hib was coated at increasing amounts (1×10^6, 5×10^6 and 1×10^7 CFU/ml), as indicated on the abscissa. Different amounts of a serum preparation containing anti-Hib were incubated, and then serum (1 μl per well in 100 μl of PiCM buffer) from an agammaglobulinemic patient as a complement source was added. C3c deposition was expressed as A_{450}. To each well 0.25 (○), 0.5 (□), 1.0 (△), or 2.0 (▽) μl of anti-Hib serum was added. Without anti-Hib serum, no complement activation was observed (★). (b) Titration of antibodies and complement. Hib was coated at 1×10^7 CFU/ml. Serial dilutions of anti-Hib antibodies were incubated for 2 h at 37°C. Next, different concentrations of serum from an agammaglobulinemic patient were incubated for 30 min at 37°C. C3c deposition was expressed as A_{450}. To each well 0.3 (□), 1.0 (△), or 3.0 (○) μl of serum from an agammaglobulinemic patient was added. No complement activation was observed without coated antigen with complement source or with coated antigen without complement source (★). Similar titration curves with TT and STAW as antigens were observed.

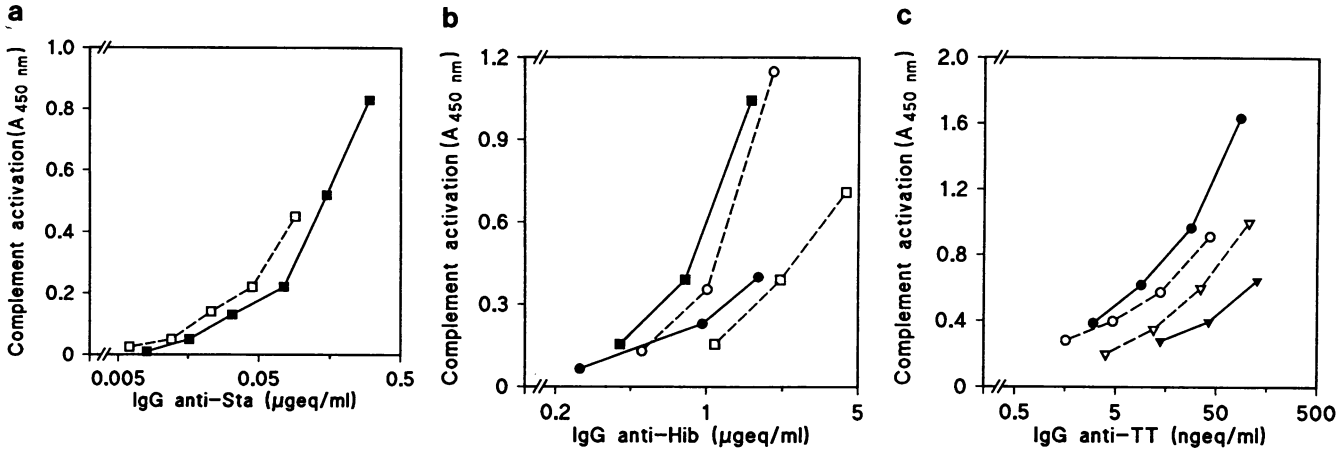

FIG. 3. (a) Complement activation by anti-STAW IgG1 and IgG2 from individual A. Serial dilutions of IgG1 (■) and IgG2 fraction, containing anti-Sta (□) were incubated for 2 h at 37°C and then incubated with serum from an agammaglobulinemic patient (1 μl per well in 100 μl of PiCM buffer). C3c deposition was expressed as A_{450}. (b) Complement activation by anti-Hib IgG1 and IgG2 from two individuals. Serial dilutions of IgG1 (■) and IgG2 fraction containing anti-Hib (□) from individual A and IgG1 (●) and anti-Hib IgG2 (○) from individual E were incubated for 2 h at 37°C and then incubated with serum from an agammaglobulinemic patient (1 μl per well in 100 μl of PiCM buffer). C3c deposition was expressed as A_{450}. (c) Complement activation by anti-TT IgG1 and IgG2 from two individuals. Serial dilutions of IgG1 (▼) and IgG2 fraction containing anti-TT (▽) from individual B and IgG1 (●) and anti-TT IgG2 (○) from individual E were incubated for 2 h at 37°C and then incubated with serum from an agammaglobulinemic patient (1 μl per well in 100 μl of PiCM buffer). C3c deposition was expressed as A_{450}.

per anti-PRP antibody, the IgG1 antibodies from four serum samples were two to seven times more active than the IgG2 antibodies from the same individual. For individual E, the opposite was found. The IgG1 preparation from individual E contained predominantly anti-PRP antibodies, in contrast to the IgG1 fractions from other individuals. However, no affinity-purified anti-PRP preparations were used to establish the relative functions of the non-anti-PRP versus the anti-PRP antibody activities.

Figure 3c shows that anti-TT IgG1 antibodies from individual B had lower C3c deposition activity than his anti-TT IgG2. Anti-TT IgG1 from individual E was about 9 times more active than his anti-TT IgG2. In Table 3, it is shown that in four of five persons, the activity of anti-TT IgG1 was one to nine times that of anti-TT IgG2.

It is important to note from Table 3 that the relative complement activation properties of IgG1 and IgG2 preparations depended on the antigen studied. Thus, in individual A anti-STAW IgG1 showed slightly lower activity than anti-STAW IgG2, whereas in the same preparation anti-Hib IgG1 and anti-TT IgG1 were more active than anti-Hib IgG2 and anti-TT IgG2, respectively. In individual E, anti-STAW IgG1 and IgG2 had equal activity, but anti-Hib IgG2 was 1.5 times more active than IgG1 and anti-TT IgG2 was less active than IgG1.

G2m(n) allotype differences and sources of complement. To

TABLE 3. Complement component C3c deposition by antibacterial IgG subclass antibodies

Individual (allotype[a])	Fraction	C3c deposition[b]		
		C3c/aSTAW	C3c/aHib	C3c/aTT
A (n^+/n^+)	IgG1	2.1 ± 0.6	0.5 ± 0.1 (0.9 ± 0.1)	1.3 ± 0.2
	IgG2	3.4 ± 0.4[c]	0.1 ± 0.0[d] (0.1 ± 0.0)[c]	1.1 ± 0.2
B (n^+/n^-)	IgG1	0.7 ± 0.2	0.7 ± 0.1 (2.8 ± 0.3)	0.6 ± 0.1
	IgG2	0.7 ± 0.2	0.1 ± 0.0[d] (0.2 ± 0.0)[c]	1.5 ± 0.2[c]
C(n^-/n^-)	IgG1	1.5 ± 0.5	0.4 ± 0.1 (3.0 ± 0.4)	1.2 ± 0.2
	IgG2	0.6 ± 0.2[c]	0.2 ± 0.0[d] (0.2 ± 0.0)[c]	0.7 ± 0.1[c]
D (n^-/n^-)	IgG1	0.6 ± 0.1	0.7 ± 0.1 (3.5 ± 0.4)	4.6 ± 0.4
	IgG2	0.5 ± 0.1	0.3 ± 0.0[d] (0.4 ± 0.0)[c]	1.0 ± 0.2[c]
E (n^-/n^-)	IgG1	3.1 ± 0.6	0.4 ± 0.1 (0.3 ± 0.0)	9.3 ± 0.9
	IgG2	2.9 ± 0.4	0.6 ± 0.1[d] (1.4 ± 0.2)[c]	1.0 ± 0.3[c]

[a] G2m(n) allotype of IgG2.

[b] Ratios (means ± standard deviations of five experiments) of C3c deposition per microgram of antibodies bound to the antigen; C3c in percentage of normal human serum, as determined with HRP-conjugated anti-C3c (based on at least three serial dilutions of the antibody fractions), and bound antibacterial antibodies in micrograms as determined with ELISA using HRP-conjugated polyclonal anti-IgG antibodies (see text). Ratios of C3c deposition per microgram of bound anti-PRP antibodies are shown in parentheses.

[c] Significantly different from IgG1 preparation by Wilcoxon matched-pair signed-rank test ($P < 0.05$) and Student's t test ($P < 0.02$).

[d] Significantly different from IgG1 preparation by Wilcoxon matched-pair signed-rank test ($P < 0.025$) and Student's t test ($P < 0.02$).

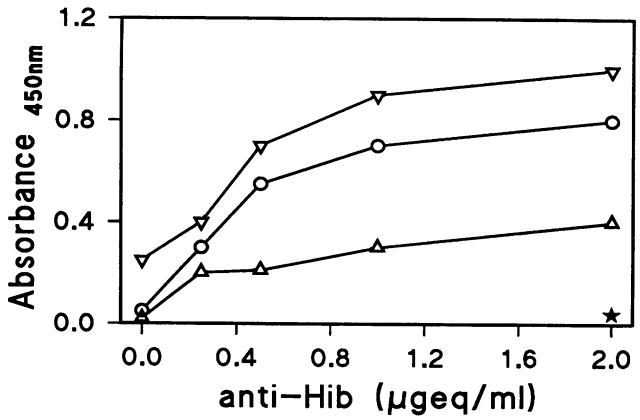

FIG. 4. Terminal complement complex (SC5b-9) formation induced by anti-Hib antibodies. The conditions were the same as for the C3c ELISA described in the legend to Fig. 2b. Mouse anti-human SC5b-9 MAb (166 ng/ml) and HRP-conjugated rabbit anti-mouse IgG (0.5 μg/ml) were used to detect terminal complement complex formation. SC5b-9 was expressed as A_{450}. To each well 1.0 (△), 3.0 (○), or 9.0 (▽) μl serum from an agammaglobulinemic patient was added. No complement activation was observed without coated antigen with complement source or with coated antigen without complement source (★).

investigate whether there might be allotype-related differences in complement activation, we used sera from individuals with different G2m(n) allotypes. Table 3 shows that differences in complement activation properties between IgG2 preparations from the five individuals were not related to their G2m(n^+/n^+) and G2m(n^-/n^-) allotypes. Six different serum samples from agammaglobulinemic patients, as sources of complement, were compared to assess possible differences in interactions with IgG1 and IgG2 antibodies. Some of the agammaglobulinemic serum samples had low levels of antibacterial antibodies, and high background levels disturbed the complement activation mediated by IgG subclass preparations. Nevertheless, no particular preference was found for any of the agammaglobulinemic serum samples, and relative differences in complement activation by IgG subclass antibodies remained similar with different complement sources (data not shown).

IgG4 antibodies. The IgG1 preparation from individual A contained antibacterial IgG4. IgG4 may serve as blocking antibody and possibly inhibit complement activation by other IgG subclasses (10, 41). However, the preparation from this individual did not show less active IgG1 than anti-Hib or anti-TT IgG2. Complement activation mediated by anti-STAW IgG1 antibodies could be inhibited only by very high concentrations of anti-STAW IgG4 antibodies from this individual.

SC5b-9 determination. We measured the formation of terminal complement complexes with the use of MAbs against SC5b-9. Figure 4 shows terminal complement complex formation by anti-Hib antibodies in the presence of different concentrations of agammaglobulinemic serum. Optimal anti-Hib dependent SC5b-9 formation and low background levels (when no anti-Hib was added) was observed with 3 μl of agammaglobulinemic serum per well (3% [vol/vol]), which is more than was needed for C3c deposition. Thus, full complement activation was established, also at a relatively low complement concentrations of 1% agammaglobulinemic serum. IgG1 and IgG2 antibodies against both bacterial antigens, STAW and Hib, were tested in this assay and the same results were obtained as in the C3c deposition ELISA (data not shown). Thus, differences between complement activation by IgG1 and IgG1 antibodies extend to the formation of the membrane attack complex.

DISCUSSION

In this study, we investigated complement activation as one of the effector functions of IgG subclass antibodies. IgG1 and IgG2 antibodies were purified from serum from healthy individuals by gel filtration and Sepharose-protein A affinity chromatography. In contrast to most other studies, in this study we separated IgG1 and IgG2 from several subjects to detect possible individual variations in activity. Complement activation by polyclonal antibodies against three different antigens was measured by ELISA. Anti-STAW IgG2 activated complement as well as anti-STAW IgG1. Anti-Hib IgG1 showed better complement activation than anti-Hib IgG2 in four individuals. In one individual, anti-Hib IgG2 was more effective in complement activation than anti-Hib IgG1. Also, anti-TT IgG1 showed better complement activation than anti-TT IgG2. However, again anti-TT IgG2 antibodies from one individual showed more effective complement activation than anti-TT IgG1.

The purification of IgG subclasses with Sepharose-protein A was performed by the method of Duhamel et al. (9) (Fig. 1). To purify IgG1 and IgG2 antibodies from an IgG pool, we used a linear pH gradient. We first purified IgG from other serum proteins with the intention of obtaining pure IgG3 after Sepharose-protein A chromatography (6). However, most IgG3 was lost in the IgA-rich fraction after gel filtration, and to assess functional properties of IgG3, larger amounts of specific IgG3 were needed. In addition, pure IgG4 could not be obtained with this procedure, as most of the IgG4 was found in the fractions that contained IgG1. IgG4 may serve as blocking antibody and possibly inhibit the complement activation by other IgG subclasses (10, 41). However, only one IgG1 preparation contained antibacterial IgG4 antibodies, and this IgG1 preparation did not show less activity than the IgG2 preparation. Apparently, the IgG4 antibody amounts were too low to interfere.

We obtained an average recovery of around 50% of the total amount of IgG1 and IgG2 subclass protein. Recoveries of specific IgG subclass antibodies against the three antigens, measured with antigen-specific IgG subclass ELISA were similar (Table 1). The total amount of purified IgG that was obtained by our procedure was relatively high compared with that obtained by Persson et al. (31), who used much less serum (0.5 to 1 ml) to separate IgG subclasses by affinity chromatography with anti-IgG subclass MAbs. Most other studies did not show recoveries of IgG subclasses or give data on recoveries of antigen-specific IgG subclasses (2, 15, 31, 43). The recovery of biological activity was good. Purification of IgG subclasses with acidic pH did not result in a loss of functional activity (2, 15, 43). All in all, this procedure is simple, allows large-scale separation of IgG1 and IgG2 antibodies, and enables the study of effector functions of purified IgG subclass antibodies against more than one antigen. When necessary, additional purification can be obtained by depletion of contaminants with MAbs against IgG subclasses. The disadvantage of loss of IgG3 antibodies in the IgA fractions may be circumvented by applying other purification strategies, such as the use of caprylic acid in the initial steps (18).

It should be realized that the *S. aureus* strain we used was

unencapsulated, protein A deficient, and not a clinical isolate. We used this strain to avoid interaction of protein A with the Fc portion of the IgG antibodies, which would interfere with specific antibody-antigen-mediated complement activation (6, 9). The results obtained with this strain do not necessarily apply to all *S. aureus* strains.

The relative amounts of C3c bound to the bacterial antigens were measured by a modified ELISA based on earlier published procedures (12, 16, 44). In our assay, the complement activation took place in the microtiter plate wells coated with whole unopsonized bacteria or bacterial antigen. We cannot exclude the possibility that coating of the bacteria to the microtiter plate, and incubation with reagents such as (low concentrations of) Tween 20 may have disturbed the integrity of the bacterial membranes. However, gram-negative organisms are relatively resistant to treatment with detergents (26). Also, we have observed that phagocytosis of intact bacteria opsonized in solution correlated very well with complement activation properties of anti-Hib (and anti-STAW) antibody (data not shown).

By detecting formation of terminal complement complexes, we showed, in an independent way, that complement is activated and that activation of C3 led to further activation of the complement cascade (Fig. 2a and 4). We used the same coating conditions in the antibody ELISA and the C3c deposition ELISA, as differences in antigen concentrations may cause variations in detection and activity of bound antibodies (11, 17).

Opsonization and complement activation by IgG subclasses has been studied extensively (2, 5, 6, 8, 14, 15, 23, 27, 34, 43). Complement activation by anti-STAW IgG subclass antibodies has not been reported earlier. Our experiments showed that anti-STAW IgG2 and IgG1 were equally active in most individuals (Table 3 and Fig. 3a). Observed statistically significant differences (Wilcoxon matched-pair signed-rank test) were small.

No earlier studies have been reported in which complement activation by anti-TT IgG2 antibodies was measured. The concentrations of anti-TT IgG2 in serum and IgG2 anti-TT in the purified fractions were very low or virtually absent in some serum samples. After correction for antibody amounts, individual differences were found in the complement activation by IgG subclasses. Anti-TT IgG1 showed an up-to-ninefold higher complement activation than IgG2 anti-TT in four individuals. In one individual, anti-TT IgG2 was more effective in complement activation than anti-TT IgG1 (Table 3 and Fig. 3c).

Antibodies against Hib have been studied in detail for their functional properties. We studied sera from healthy persons before any vaccination with Hib antigens and measured bound antibodies and C3c deposition on whole bacteria. In addition, the contribution of anti-capsular polysaccharide (PRP) antibodies was examined separately after anti-PRP specific ELISA. Our findings obtained by comparing complement activity by anti-Hib IgG1 and IgG2, measured with whole-cell ELISA were not different from activity by anti-PRP, measured with anti-PRP ELISA (Table 3). In healthy unvaccinated donors, both anti-outer membrane protein antibodies and anti-capsular polysaccharide (PRP) antibodies play a role in the host defense against Hib (19, 21). After vaccination with PRP conjugates, the anti-PRP antibodies may dominate in the effector functions (21).

Most studies on the effector functions of anti-Hib antibodies included complement activation via the alternative pathway and consequently used much higher concentrations of serum (up to 20%) as complement source (2, 7, 19). We used 1% agammaglobulinemic serum and found this sufficient for classical pathway activation. Upon using higher concentrations of agammaglobulinemic serum as a source of complement, high background levels were found, which were caused by low levels of antibacterial antibodies still present in the agammaglobulinemic serum.

Weinberg et al. (43) prepared IgG1- and IgG2-depleted fractions from IgG pools obtained from adults immunized with the Hib capsular polysaccharide PRP vaccine. No significant differences were found in the ability to activate complement-mediated bacteriolysis or in protection of infant rats. More recently, anti-PRP IgG2 affinity-purified subclass fractions from pooled sera were shown to be less effective than anti-PRP IgG1 in a complement-mediated bactericidal assay and in opsonic activity (2). In addition, anti-PRP IgG1 from all individuals showed more bactericidal activity than their anti-PRP IgG2; however, some IgG2 preparations were as active as IgG1 preparations, when different individuals were compared (2). Our findings show that in most individuals, anti-Hib IgG1 is more active than anti-Hib IgG2 and that in some individuals the reverse can be found. We thus confirm that anti-Hib IgG subclass antibodies are heterogeneous with respect to complement activation and that IgG2 can certainly be effective against Hib. Although we realize that only (affinity-) purified anti-PRP and anti-"non-PRP" antibody preparations could further resolve the relative IgG subclass activities, our results and those of others strongly suggest that antibody specificity (19, 21) and possibly the functional affinity (17, 20) of the antibody, rather than the subclass, determines the effectiveness of anti-Hib.

In conclusion, we have found that anti-STAW IgG2 is an effective antibody in defense against this microorganism. IgG2 antibodies against Hib and TT showed interindividual differences: one IgG2 preparation showed better complement activation than IgG1, but other preparations showed less complement activation. Thus, the relative complement activation by IgG subclass antibodies is not strictly related to the subclass. Other factors, such as epitope density (14, 27), antigenic specificity (19, 21), and affinity (4, 13, 17, 20, 36) of the antibody, seem to be of more importance to the relative effectiveness of IgG1 and IgG2 antibodies. The observed interindividual differences in functional activity of the IgG subclasses has implications for the evaluation of early phase 1 studies of new vaccines. Effector functions, such as complement activation and opsonization should be measured for serum from each individual separately, rather than for pooled serum samples in which interindividual variations are masked.

With respect to the clinical significance of IgG2 deficiencies, our results indicate that IgG2 antibodies may certainly contribute to the immune defense. General conclusions with regard to IgG2 effector functions have to be drawn with great care for the abovementioned reasons. The defense against bacteria, however, involves not only complement activation but also phagocytosis. Further studies to analyze the effector functions of our IgG subclass preparations in phagocytosis assays are under way.

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