Human Immunoglobulin G Subclass Immune Response to Outer Membrane Antigens in Meningococcal Group B Vaccine

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The immunoglobulin G (IgG) subclass distribution of antibodies against the major outer membrane proteins from serotype 2a *Neisseria meningitidis* in human vaccinees was studied by immunoblotting. The volunteers received two doses of a noncovalent complex of group B polysaccharide and outer membrane material from the same meningococcal strain. Six weeks after the first vaccination the antibodies mounted against the class 1 and 5 proteins belonged mainly to the IgG1 and IgG3 subclasses. However, the binding of IgG3 to the class 5 proteins decreased markedly in serum samples taken after 25 weeks. Antibody binding to the serotype-specific class 2 protein was dependent on renaturation of the antigen by a dipolar ionic detergent (R. E. Mandrell and W. D. Zollinger, *J. Immunol. Methods* 67:1-11, 1984). The immune response against this protein showed more individual variation and consisted of IgG1 or IgG3 or both, often combined with IgG4.

The human antibody response after the meningococcal vaccine trial in Norway in 1981 and 1982 with a noncovalent complex of group B polysaccharide and outer membrane vesicles (OMV) from serotype 2a *Neisseria meningitidis* (7) has been studied in several ways. The antibody class levels were determined by enzyme-linked immunosorbent assay (17), the bactericidal titers were evaluated with baby rabbit (7) and human complement (L. O. Frøholm, unpublished data), and the binding patterns of immunoglobulin G (IgG), IgA, and IgM antibodies to the major outer membrane proteins in OMV were studied by immunoblotting (23, 24). The latter studies showed considerable individual variations in antibody binding to these proteins, the meningococcal class 1, 2, and 5 proteins (6). Most vaccinees had antibodies directed to the class 5 proteins, whereas the class 2 proteins bound antibodies only weakly. Moreover, the analysis suggested a correlation between high serum bactericidal activity and distinct IgG binding to the class 1 protein. Complement-mediated bactericidal activity is thought to be the main defense mechanism against meningococcal infections (20). As the IgG subclasses differ in their ability to fix complement and also in binding to phagocyte cells (11, 18, 19), it was of interest to study the distribution of the IgG subclasses raised in the volunteers against the various antigens in the vaccine. Also, we wanted to see whether the immune response to the meningococcal membrane proteins was restricted to one or a few subclasses, as is the case for other immunogens (18).

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MATERIALS AND METHODS

Vaccination sera. The vaccine trial has been described previously in detail (7). Briefly, 33 human adults were vaccinated twice at an interval of 4 weeks with OMV from *N. meningitidis* M986 NCV-1 serotype 2a and group B polysaccharide, while 27 volunteers received the placebo vaccine. Ten serum samples with the highest bactericidal titers, determined with human complement (range, 1:>1,024 to 1:32) (Frøholm, unpublished data) taken 6 weeks after the

first vaccination, were selected for the present study. As the previous immunoblotting analysis indicated that meningococcal carriers had antibodies against an antigen of molecular weight 22,000 (23, 24), vaccinee 80, who became a carrier between weeks 6 and 25, and one placebo control (no. 3), who became a carrier 4 weeks after the first vaccination, were also studied. The bactericidal titers for these two sera were 1:1 and 1:4, respectively. The volunteer numbers are the same as reported previously (7, 23, 24). The sera were stored at -25°C.

Gel electrophoresis and immunoblotting. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12) was carried out in 12% acrylamide gels as described (23). OMV, prepared from the same meningococcal strain as the vaccine OMV antigen, was boiled for 5 min in sample buffer with sodium dodecyl sulfate and 2-mercaptoethanol. The electrophoresis was performed in gel slabs (14 by 12 cm, 1.5 mm thick) or in minislabs (7 by 8 cm, 0.75 mm thick). The gels were blotted to nitrocellulose filters (pore size, 0.45 µm) at 0.4 A for 2.5 h in cold transfer buffer by the method of Towbin et al. (22). In some experiments a final concentration of 0.2% of the detergent Empigen BB (Albright & Wilson, Whitehaven, Cumbria, United Kingdom) was added to the transfer buffer to renature the class 2 protein of OMV (14). Preliminary experiments showed that when 0.1% Empigen BB was present in the separating gel (14), less sharp bands in the gels and on the corresponding immunoblots and only slightly increased IgG binding was observed. Therefore, the detergent was only used in the transfer buffer. After electroblotting, the nitrocellulose filters were cut into 4-mm-wide strips, each corresponding to approximately 5 µg of OMV protein, before gel electrophoresis. Guide strips were stained with amido black (8). Strips blotted in the presence of Empigen BB were difficult to destain.

In the following procedure, 3% bovine serum albumin in physiological saline was used as the blocking and diluting agent and physiological saline was used for washing. After blocking for 30 min, the strips were incubated overnight in a 1:200 dilution of the postvaccination sera and washed four times, and individual strips were incubated for 2 h with anti-human IgG subclass antisera raised in rabbits (15). The following subclass antisera were used: anti-IgG1 (E4#7), anti-Ig2 (B5a#4), anti-IgG4 (K5#8-12), all diluted 1:200, and

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FIG. 1. IgG subclass binding of individual 6-week postvaccination sera to the meningococcal class 1 protein. The height of the bars represents the staining intensity of IgG subclass binding to the protein, rated as weak (+1) to strong (+4) on the vertical axis. The volunteer numbers are marked on the horizontal axis under the corresponding bars. The electroblotting was performed with the detergent Empigen BB. Volunteer 3 received the placebo vaccine, but became a meningococcal carrier between the first and second vaccination.

anti-IgG3 (284) diluted 1:2,000. The dilutions of the subclass antisera were chosen to give the same optical density when tested in microtiter plates coated with isolated myeloma proteins of the different subclasses (15). After being washed, the strips were incubated for 2 h with a 1:1,000 dilution of sheep anti-rabbit IgG antibodies, conjugated to horseradish peroxidase, and washed, and the binding of antibodies was detected as reported (23). Strips incubated with the same subclass antibody were stained for similar times. Control strips, treated as described above, were included in each experiment, but without the postvaccination serum in the overnight incubation. These strips showed no or negligible antibody binding to the major membrane proteins.

In some experiments blotted strips were oxidized with 10 mM sodium periodate in 0.05 M sodium acetate buffer, pH 5.0, by a modification of the method of Woodward et al. (25). The oxidation was performed in the dark for 30 min at room temperature. The strips were then washed briefly in physiological saline before blocking as reported above. Our studies showed that these conditions abolished antibody binding to the lipopolysaccharide region on the blots.

The staining intensity following antibody binding to each of the major membrane proteins on the blots was rated visually.

RESULTS

IgG subclass binding to the class 1, 2, and 5 meningococcal major outer membrane proteins. The binding of the different IgG subclass antibodies in 6-week postvaccination serum samples to the major outer membrane proteins was studied

FIG. 2. IgG subclass binding of individual 6-week postvaccination sera to the meningococcal class 2 protein. See the legend to Fig. 1 for details.

with and without Empigen BB in the transfer buffer. The molecular weights of the class 1, 2, and 5 proteins from strain M986 were previously reported to be 43,000, 41,000, and 30,000, respectively (23). In the absence of detergent, IgG1 antibodies or IgG1 and IgG3 antibodies bound to the class 1 protein (Fig. 1). Two vaccinees (nos. 71 and 111) also showed distinct IgG2 binding. Periodate oxidation of the blotted strips had no effect on the binding of IgG2 in these two sera. When Empigen BB was present during electroblotting, the patterns of antibody binding to the class 1 protein were generally unchanged, except for the appearance of IgG3 binding with the serum from volunteers 3, 52, and 67.

TABLE 1. Strength of IgG subclass binding to meningococcal class 5 proteins with 6- and 25-week postvaccination sera^a

Volunteer no.	IgG binding			
	6-wk sera		25-wk sera	
	IgG1	IgG3	IgG1	IgG3
2	++(+)	++++	++	(+)
17	++	++++	++	0
42	++	++(+)	++	(+)
52	++	++++	++	0
67	++(+)	++++	+	+
71	+	++	(+)	(+)
80	++	+++(+)	++	0
87	+++	++++	++	0
101	++	+	+	(+)
111	++	++(+)	+ ^b	0 ^b
112	++	++	++	+
3 ^c	++(+)	+	++	+

^a The strength of antibody binding was rated as weak (+) to strong (++++). The electroblotting was performed without Empigen BB in the transfer buffer.

^b This serum was drawn after 77 weeks.

^c This individual was a placebo control who became a meningococcal carrier 4 weeks after vaccination.

FIG. 3. Immunoblots showing the time-related decrease in IgG binding to the meningococcal class 5 protein. Binding of subclasses IgG1 (lanes 1) and IgG3 (lanes 3) in sera from volunteers 2, 17, and 87 taken 6 weeks (lanes a) and 25 weeks (lanes b) after vaccination is shown. The arrows to the left mark the positions of the class 1, 2, and 5 proteins. The band near the top of the strips corresponds to the antigen of molecular weight 180,000. The blotting was performed without Empigen BB in the transfer buffer. Antibody binding was detected with peroxidase-conjugated antibodies.

IgG subclass binding to the serotype-specific class 2 protein is shown in Fig. 2. All individuals had some antibody activity against this protein, but the response was heterogeneous. Whereas IgG1 binding to the class 2 protein was relatively distinct when the electrotransfer was performed without Empigen BB but increased in its presence, binding of IgG3 and IgG4 was only observed with the detergent.

The IgG1 and IgG3 subclasses bound to the class 5 proteins (Table 1). The binding patterns were unaffected by Empigen BB. Previously, it was demonstrated that the IgG antibody response against the class 5 proteins decreased between the 6th and 25th week after vaccination (23). Therefore, the IgG1 and IgG3 subclass binding of the 6-week postvaccination sera was compared with that of the 25-week sera. The results (Table 1 and Fig. 3) show that in contrast to IgG1, the binding of IgG3 to the class 5 proteins decreased markedly with time after vaccination. A smaller time-dependent decrease of IgG3 binding to the other OMV proteins was observed (Fig. 3).

IgG subclass binding to other OMV components. In the prior study it was found that many of the vaccinees, in addition to IgG antibody activity to the major outer membrane proteins, also had antibodies directed against other OMV components (23, 24). The IgG class and subclass binding of a 6-week postvaccination serum to OMV blotted in the presence and absence of Empigen BB is shown in Fig. 4. Without detergent, immunoreactive bands corresponding to molecular weights of 180,000, 86,000, 43,000 (class 1 protein), 34,000 (class 4 protein), and 30,000 (class 5 proteins) were observed. In the presence of detergent IgG binding to the class 2 protein increased markedly, whereas the bands of molecular weights 180,000 and 86,000 disappeared. Generally, the antibody activity in the vaccinees against the 180,000-molecular-weight antigen consisted of

FIG. 4. Immunoblot analyses of IgG antibody class and subclass binding of the 6-week postvaccination serum from volunteer 87 to OMV electroblotted in the absence (A) and presence (B) of Empigen BB. The OMV proteins were separated in 14- by 12-cm gels (A) or in 7- by 8-cm gels (B). The lanes marked IgG refer to the binding of human IgG class antibodies, while the other lanes show the binding of the subsequently applied rabbit anti-human IgG subclass sera. Antibody binding was detected with peroxidase-conjugated antibodies. The arrows mark the class 1, 2, and 5 proteins. Note the increased antigenicity of class 2 proteins in the presence of Empigen BB, but the absence of the immunoreactive bands of molecular weights 180,000 (180k) and 86,000 (86k).

IgG1 or IgG3 or both, whereas the 86,000-molecular weight antigen showed weak IgG1 binding.

The IgG antibodies against the antigen of molecular weight 22,000, possibly associated with carriage of meningococci by the volunteers (23, 24), were mainly of subclass IgG1 or IgG3. The binding of these antibodies was not affected by periodate oxidation of the blots. However, Empigen BB abolished antibody binding to this antigen.

The IgG response in postvaccination serum samples to the lipopolysaccharide region was restricted to subclass IgG2, and the binding was abolished by periodate oxidation of the blots.

DISCUSSION

This report describes the IgG1, IgG2, IgG3, and IgG4 subclass response to a meningococcal vaccine in human volunteers. The four IgG subclasses are different in terms of structure, serum concentrations, and functions, and the response to certain antigens may be restricted to one or few of the subclasses (18). Binding of the IgG subclasses to the blotted OMV components was detected with polyclonal anti-human IgG subclass antibodies raised in rabbits. These sera are specific and show little or no cross-reaction, as demonstrated by direct binding as well as inhibition in sensitive enzyme-linked immunosorbent assay and hemagglutination systems (15).

The experimental conditions used in the immunoblotting technique can lead to loss of antigenic determinants, caused by, e.g., sodium dodecyl sulfate and heat treatment. Since sodium dodecyl sulfate is partly removed during electroblotting, antigen renaturation may to some extent take place on the nitrocellulose filter. Also, the method we used to score the strength of antibody binding to the individual antigens is not strictly quantitative. With these limitations in mind, the results provide a measure of the relative subclass antibody levels to the vaccine components in the volunteers. The staining intensity of the IgG class binding to the major outer membrane proteins observed previously (23, 24) correlated well with that of one or more of the IgG subclasses. The use of polyvalent sera to detect IgG subclass binding may have the advantage over monoclonal reagents in that the signals on the blots are likely to be stronger, since the former antibodies are reacting with more than one epitope on the subclass molecule.

The serotype-specific class 2 protein is heat labile and represents in its trimer form the porin in the meningococcal membrane (6). In the previous studies we observed that after heat treatment, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotting of the OMV, the antibodies in the postvaccination sera bound only weakly to the monomeric protein (23, 24). However, Mandrell and Zollinger (14) demonstrated that the antigenicity of this heat-denatured protein was potentiated when the dipolar ionic alkyltrimethylbetaine detergent Empigen BB was used in the electroblotting buffer. The detergent probably partly renatured the protein due to its structural resemblance to membrane phospholipids (9). We did not observe strong IgG subclass binding to the class 2 protein with all sera (Fig. 2), suggesting either that the serotype protein was not a good immunogen in all individuals or that the experimental conditions were not optimal for antibody binding. As nearly half of the volunteers showed a distinct antibody response, the antigen was probably sufficiently renatured, and individual variations in the immune response to the vaccine are a more likely explanation.

The results indicated that the epitopes on the class 2 protein that bound IgG3 and IgG4 were dependent on Empigen BB, whereas those that bound subclass IgG1 showed some epitope expression without detergent. Probably, the determinants on the antigen that elicited the IgG3 and IgG4 responses were conformational and destroyed by the experimental conditions, whereas those that gave rise to the IgG1 antibodies were more stable or more easily renatured. The same explanation may hold for IgG3 binding to the class 1 protein observed for a few sera in the presence of detergent. However, Empigen BB seemed to abolish or diminish antibody binding to the antigens of molecular weights 180,000 (previously reported to have a molecular weight of 140,000 [23]), 86,000, 22,000 and the lipopolysaccharide. Detergents have been reported to remove proteins from nitrocellulose filters (13). Therefore, as suggested by Mandrell and Zollinger (14), immunoblotting studies have to be performed both with and without Empigen BB in the transfer buffer.

The antibody-binding patterns of the class 1 and 5 proteins showed fewer individual variations than the class 2 protein. The strong IgG binding to the class 1 protein, previously demonstrated to correlate with high bactericidal activity of the postvaccination sera (23), consisted of IgG1 and IgG3. The distinct IgG2 binding observed with two sera (Fig. 2) was not affected by periodate oxidation of the antigen, although IgG2 antibodies are mainly directed against carbohydrates (16, 26). The antibodies against the class 5 proteins were also restricted to the IgG1 and IgG3 types (Table 1). The time-related decrease in IgG binding to these proteins (23) seemed to be caused by a fall in the binding of IgG3. This result may be explained by the higher catabolic rate of the IgG3 than of the other IgG subclasses (18). A less marked decrease was noted for IgG3 binding to the other OMV components (Fig. 3). The distinct IgG3 signal observed against several vaccine antigens argues against the use of staphylococcal protein A to detect antibody binding on blots. IgG3 is the only human IgG subclass that does not bind to protein A (10).

Volunteer 3, receiving the placebo vaccine, had some antibody activity against the class 1, 2, and 5 proteins (Fig. 1 and 2, Table 1). This was probably caused by the recent meningococcal carrier status of this individual. In the prior studies, volunteers 52 and 67 were the only ones with high serum bactericidal titers who did not show strong IgG binding to the class 1 protein (23). However, in the presence of detergent these two vaccinees had IgG3 activity against this protein and a good response against the class 2 protein (Fig. 1 and 2), which might explain the high bactericidal titers.

Complement-dependent bactericidal activity is believed to be the main immune defense against meningococcal infections (20). Bactericidal antibodies directed against the class 1, 2, and 5 proteins have been demonstrated (2, 4, 5, 21, 27). Other proteins in the OMV may also give rise to such antibodies (3). The ability of the different IgG subclasses to fix complement differ markedly. IgG3 and IgG1 antibodies are the most effective, IgG2 has lower activity, and IgG4 has no activity (1). Phagocytosis, although probably less important in defending meningococcal infections (20), also shows IgG subclass specificities; the IgG1 and IgG3 subclasses demonstrate the strongest binding to the Fc receptors on monocytes and polymorphonuclear leukocytes (11, 18, 19). Our immunoblotting study indicated that the IgG response against the major meningococcal outer membrane proteins in sera of high bactericidal activity were mainly of the sub-

classes IgG1 and IgG3. This implies that the vaccine had given rise to those IgG subclasses that are the most effective in activating complement and in phagocyte binding.

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