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Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Subclass Antibodies Specific for Enterobacterial Re Core Glycolipid in Healthy Individuals and in Patients Infected by Gram-Negative Bacteria

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An enzyme-linked immunosorbent assay was developed to study the subclass distribution of immunoglobulin G (IgG) specific to the core glycolipid (CGL) of the Re mutant of *Salmonella minnesota* R595 in serum samples from individuals with an IgG response toward these antigens. In a group of healthy blood donors, we detected predominantly the IgG2 and IgG1 subclasses. In a group of patients in an intensive care unit who developed infectious complications due to gram-negative bacteria, the anti-CGL IgG activity was due mainly to the IgG2 and IgG3 subclasses. In all serum samples found to be IgG positive, the assay for anti-CGL IgG2 was positive. This subclass was revealed to play a predominant role in patients displaying a seroconversion or a significant rise in their antibody response toward CGL. IgG4 was found or appeared only in patients with confirmed bacteremia. In addition, we observed a drop in anti-CGL IgG2 before the death of patients undergoing a septic shock or an irreversible organ failure, suggesting that the anti-CGL IgG2 activity could be used as a marker of the evolution of the illness in this group of patients.

It is now accepted that acquired antibody responses toward given antigens may be limited to the preponderant production of one or certain determined immunoglobulin G (IgG) subclasses, with some biological activities controlled by the constant region (Fc fragment) of IgG varying from one subclass to another. On the other hand, Oxelius (11) and Schur et al. (13) have demonstrated that a deficiency of an IgG subclass directed toward polysaccharides may be associated with increased susceptibility to certain infections such as bronchopulmonary infections due to *Haemophilus influenzae* type b.

Relatively few studies have been performed to quantitate the IgG subclass distribution of antibodies with a specific activity toward a given antigen. More particularly, carbohydrate antigens have been shown to elicit an immune response that is restricted to certain subclasses. Indeed, Yount et al. (19) have reported that in volunteers injected with dextran and levan, they detected only IgG2 antibodies against these antigens, even after periods of immunization as long as several years. Similarly, antibodies to hemophilic factor VIII appear to be restricted to the IgG4 subclass (1, 14), and anti-Rh antibodies are predominantly of the IgG1 subclass (18). On the other hand, Morell et al. (9) have shown that antibodies toward hepatitis B surface antigen are predominantly of the IgG1 and IgG3 subclasses, as they are in the case of other viral infections such as those caused by rubella virus (6), cytomegalovirus (7), and respiratory syncytial virus (4).

Lipopolysaccharides play a major role in the host-parasite relationships involving gram-negative pathogens. The O-specific side chains of lipopolysaccharides are extremely heterogeneous, and as a consequence, anti-O antibodies are type specific. The inner core region (core glycolipid [CGL]) and lipid A are structurally and antigenically similar in lipopolysaccharides from most members of the family *Enterobacteriaceae*. Antibodies toward CGL or lipid A may thus cross-react with various gram-negative bacteria.

In a previous study of antibody responses to enterobacterial Re CGL and lipid A in patients infected with gram-negative bacteria (10), we found that a stable positivity or a significant increase of their total anti-CGL IgG activity was correlated with a favorable outcome.

The recent development of monoclonal antibodies specific against the different human IgG subclasses has offered the opportunity to improve the specific detection of antibody responses due to the different subclasses. In the present study, to improve our knowledge of the serological response toward gram-negative bacteria, we decided to investigate the IgG subclass response toward the CGL from *Salmonella minnesota* R595 in healthy individuals and in patients infected with gram-negative bacteria. Therefore, we developed an enzyme-linked immunosorbent assay (ELISA) method in which four monoclonal antibodies specific against IgG subclasses were used as markers of the serological response to the antigen. In addition, in patients who developed severe infections caused by gram-negative bacteria, we looked for correlations, if any, between anti-CGL IgG subclass distribution and clinical evolution.

MATERIALS AND METHODS

Antigen. The procedure for the preparation, characterization, and purification of the CGL from *S. minnesota* R595 has been previously described (10). Briefly, CGL was extracted from *S. minnesota* R595 by the phenol-chloroform-petroleum ether method (3). Finally, the CGL antigen was characterized and purified by gel chromatography by the method described by Young et al. (18).

Human sera. Serum samples were obtained from 43 patients who developed severe gram-negative infections, either focal or bacteremic. These patients were selected from a

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group of high-risk patients in an intensive care unit and had the following predisposing conditions: (i) complicated abdominal surgery (19 patients), including laparotomy for peritonitis, fistulae, or perforations; repeated abdominal operations; or surgery for severe necroticohemorrhagic pancreatitis; (ii) severe multiple trauma (6 patients) involving two or more anatomical regions and requiring mechanical ventilation for more than 24 h; (iii) complicated cardiac or vascular surgery (13 patients), including emergency operations for abdominal aortic aneurysms and aortocoronary bypass followed by complications requiring mechanical ventilation for at least 7 days; or (iv) different infections such as bronchopneumonia, encephalitis, chronic bronchitis, and pneumonia (5 patients).

The patients were observed for the occurrence of severe infectious episodes with signs of systemic involvement, such as febrile spikes above 38.5°C and the presence of more than 12,000 leukocytes per μl. Moreover, the diagnosis of respiratory tract infection required stethacoustical and radiological signs of lung involvement, as well as production of purulent sputum. The diagnosis of intraabdominal infection required the presence of a purulent collection, as demonstrated either by echography or laparotomy. Blood and other appropriate samples were collected for culture.

Death was attributed to the infectious episode if it occurred during irreversible septic shock or when infection caused irreversible, often multiple, organ failure leading to death.

Serum samples were taken from these patients on admission and thereafter every 7 days. In the present study, we selected specimens in which variations in the anti-CGL antigen IgG activities were observed in the anti-CGL ELISA previously described (10).

In addition, we investigated serum specimens from healthy subjects who were found to be IgG positive toward the CGL antigen or negative, as determined by ELISA.

Purification of the human immunoglobulin subclasses. Human myeloma proteins IgG1, IgG2, IgG3, and IgG4 were purified from plasma by the technique described by Robbin and Wistar (12) and were kindly supplied by A. Jeanson (Unité de Production d'Anticorps Monoclonaux, Université de Liège). IgG1 and IgG3 are κ light chain-type immunoglobulins, whereas IgG2 and IgG4 have a λ light chain.

Murine monoclonal antibodies to human IgG subclasses. Anti-IgG subclass monoclonal antibodies (UNIPATH, Belford, United Kingdom) were in the form of ascitic fluids produced from the clones JL512, GOM1, and RJ4 for IgG1, IgG2, and IgG4, respectively. Anti-IgG3 was produced and kindly provided by J. M. Cloes (Unité de Production d'Anticorps Monoclonaux, Université de Liège) from the clone 28.5.H11. This anti-IgG3 monoclonal antibody was revealed to be active in the ELISA toward IgG3 (κ light chain) from three different origins (MET, CF, and REY; provided by the Institute for Cancer Research, Berne, Switzerland); and its specificity was assessed by the total absence of activity toward a panel of myeloma proteins such as IgM, IgA, IgG1, IgG2, IgG4 and isolated human κ or λ chains as well as toward human serum albumin. All the monoclonal antibodies were of the IgG1 isotype.

Detection of anti-CGL IgG subclasses by ELISA: general procedure. ELISA was carried out in polystyrene microplates (Immunoplate II; Nunc, Roskilde, Denmark) by a method derived from that of Voller et al. (17), which was slightly modified as described previously (10).

Briefly, for each serum sample, four rows of a plate coated with 50 μl of a solution of 10 μg of CGL antigen per ml were diluted in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) containing 0.02% (wt/vol) NaN_3 (coating buffer). After one night at 4°C, the plates were washed five times with phosphate-buffered saline (pH 7.5)–0.05% (vol/vol) Tween 20 (washing buffer) and were drip-dried. After that 0.1 ml of 2% fetal bovine serum in phosphate-buffered saline–Tween (contact buffer) was added to prevent nonspecific binding of proteins, and after 30 min the plates were washed. Twofold dilutions of sera from the patients in contact buffer were distributed four times in the rows of the plates (0.05 ml from 1:4 to 1:512). After 2 h at 20°C, 0.05 ml of each anti-human IgG1, IgG2, IgG3, or IgG4 was added at dilutions of 1:500 for anti-IgG1 and anti-IgG2, 1:5,000 for anti IgG3, and 1:16,000 for anti IgG4 (for the optimal dilution, see below). Dilutions were made in contact buffer. The plates were incubated for 2 h at 20°C and then washed with phosphate-buffered saline–Tween. Thereafter, 0.05-ml samples of alkaline phosphatase-coupled goat anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.) diluted at 1:500 in contact buffer, i.e., a standard value commonly used in our test, were distributed. The plates were again kept for 2 h at 20°C and then washed and dried. Samples (0.05 ml) of a fresh solution of 4-nitrophenyl phosphate (1 mg/ml in diethanolamine buffer) were added, and the plates were kept at 37°C for 1 h. Finally, the enzyme reaction was stopped by adding 0.15 ml of 0.4 M NaOH.

The specific antibody activity was monitored at 405 nm with a multichannel spectrophotometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.). A serum sample was considered to have a specific activity when the optical density (OD) was ≥0.3 at a dilution of 1:4 and ≥0.2 at the dilutions given below. Generally, the mode of expression of the results was semiquantitative. The symbols (+), +, ++, and +++ indicate absorbances of ≥0.3, ≥0.5, ≥1.0, and ≥1.5, respectively.

In the ELISA for total anti-CGL IgG activity or in certain serum samples for subclass determinations, activities were expressed in terms of the endpoint titration given by the maximum dilution that gave an OD value that was significantly higher than that of a control serum sample without antigen. We considered negative those sera that gave a maximum active dilution of <1:200. The different ELISA curves obtained for each subclass activity were optimized by the program described by Feldman and Rodbard (2).

Standardization of the assay and control of subclass specificity. The different human IgG subclasses that were purified were diluted at a concentration of 0.1 μg/ml and were used directly as the antigen. The assays were performed by using a checkerboard titration with twofold dilutions of each monoclonal antibody at dilutions that varied from 1:250 to 1:502,000. Optimal dilutions of the monoclonal antibodies, defined as those giving an absorbance of <0.3 in the absence of antigen and a specific OD value of about 1.2 with positive sera chosen as a standard, were 1:500 for anti-IgG1 and anti-IgG2, 1:5,000 for anti-IgG3, and 1:16,000 for anti-IgG4.

Because the specific activity of some monoclonal antibodies has been shown to be influenced by different assay systems (5), we evaluated the specificity of the assay for each subclass by measuring the activity of each monoclonal antibody toward the various subclasses. In addition, we looked for the influence of the whole IgG subclasses on the activity of one of them by measuring activities of serum samples before and after fractions were pooled with sera displaying one or more elevated subclass activities toward the antigen.

TABLE 1. Distribution of anti-CGL subclass activities in serum samples from 16 positive and 4 negative healthy blood donors

Donor	Sex[a]	Anti-CGL IgG activity	Activity of the following subclass[b]:		
			IgG1	IgG2	IgG3
1	M	1:3,200	+++	++	+++
2	F	1:400	+	+	
3	F	1:400	(+)	+	(+)
4	M	1:400	+	+	
5	M	1:800	+	+	(+)
6	M	1:800	++	++	(+)
7	F	1:200		+	
8	M	1:200		+	
9	M	1:400		+	
10	M	1:200		(+)	
11	M	<1:200			
12	M	1:400		+	
13	M	1:800	+	++	
14	F	1:1,600		+++	(+)
15	M	1:400		+	
16	M	1:400		++	(+)
17	F	<1:200	(+)		
18	F	<1:200			
19	M	<1:200			
20	M	1:3,200	++	+++	

[a] M, Male; F, female.
[b] Negative values are not reported. All donors were negative for IgG4. See text for definitions of symbols.

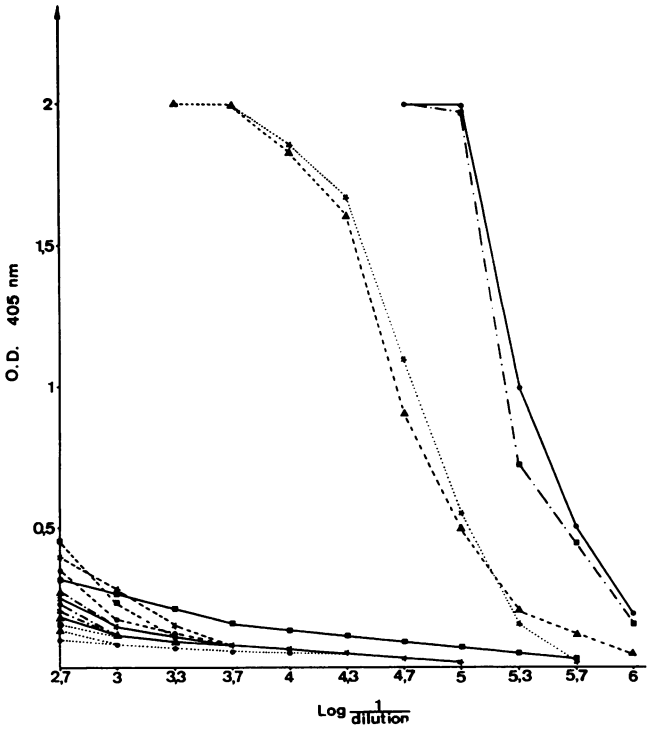

FIG. 1. Anti-human IgG subclass activities of monoclonal antibodies anti-IgG1 (●), anti-IgG2 (▲), anti-IgG3 (■), and anti-IgG4 (★) in an assay in which each human IgG1 (———), IgG2 (— — — —), IgG3 (—·—·—), and IgG4 (·····) was used as an antigen. Ordinate, OD at 405 nm; abscissa, dilutions of the monoclonal antibodies (logarithmic scale).

RESULTS

Determination of subclass specificity and reproducibility of the test. The activity of each monoclonal antibody was tested by establishing titration curves in assays in which IgG1, IgG2, IgG3, and IgG4 were used as antigens and coated in the plates at 0.5 μg/ml. We did not observe any significant cross-reactivity between a human IgG subclass and the other IgG subclasses at a monoclonal antibody dilution at which each IgG subclass was efficiently recognized by its related antibody, allowing us to assess for each subclass the specificity of the assay (Fig. 1).

In addition, several assays were performed by using sera with elevated anti-CGL activities due to one or two subclasses, which were investigated before and after they were mixed in the same proportions, to look for a possible competition effect between the whole subclass population and a defined subclass for the antigen. In all assays, the

TABLE 2. Distribution of anti-CGL IgG subclass activities in patients on admission

Predisposing condition	No. of patients:		Distribution (no.) of activities of the following subclasses:			
	IgG positive[a]	IgG negative	IgG1	IgG2	IgG3	IgG4
Multiple trauma	6	0	0	6	2	0
Complicated abdominal surgery	18	1	3	18	7	2
Complicated vascular surgery	13	0	0	13	6	3
Diverse infections	4	1	1	4	3	0

[a] Patients with a maximum active dilution of ≥1:200.

TABLE 3. Evolution of anti-CGL IgG activity and distribution of subclass activities related to the clinical outcome[a]

Predisposing condition	No. of patients with the following IgG activities:			Distribution (no.) of activities of the following subclasses:				No. of deaths
	Increasing	Stable	Decreasing	IgG1	IgG2	IgG3	IgG4	
Multiple trauma	6	0	0		6	4		0
Complicated abdominal surgery	11	1	7	5	15	8	3	9[b]
Complicated vascular surgery	8	0	5	1	11	7	5	5
Diverse infections	5	0	0	3	5	5	1	0

[a] The patient population was the same as that for which data are presented in Table 2.
[b] One patient displaying an increase of activity died due to a heart failure.

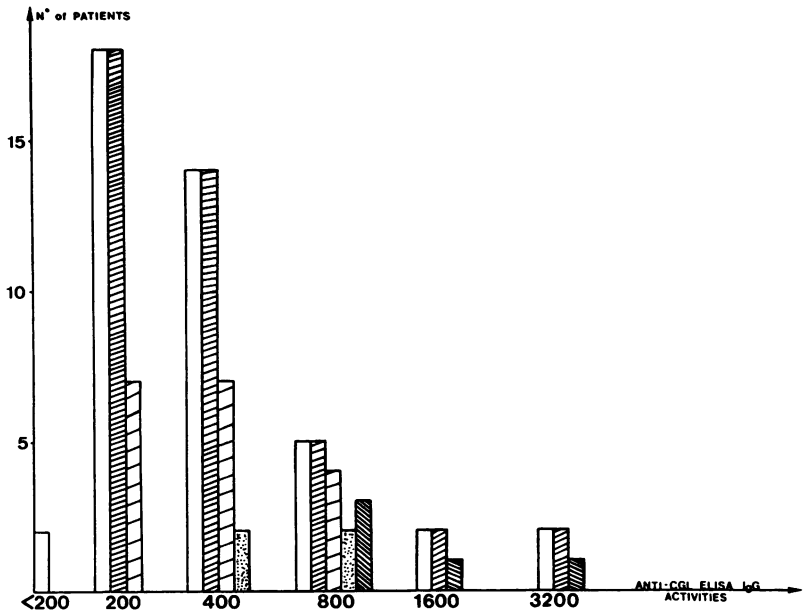

FIG. 2. Distribution of patients found to be CGL IgG positive by ELISA on admission and their IgG subclass activities, according to their total IgG activity, as measured by ELISA, in terms of maximum active dilutions (see text). Ordinate, number of patients; abscissa, total IgG ELISA activity. Symbols: □, number of patients with a total IgG activity; ▨, number of patients that were IgG1 positive; ▨, number of patients that were IgG2 positive; ▨, number of patients that were IgG3 positive; ▨, number of patients that were IgG4 positive.

activity of each subclass was not significantly altered by the presence of the other subclasses. These control experiments were repeated with other mixtures of serum samples. By these assays, we never detected any competition effect. The value obtained for each subclass activity may therefore be considered intrinsic.

On the other hand, the reproducibility of the test was checked by repeated testing. The coefficients of variation of the optimal ODs were determined and found to be equal to 5.1% for IgG1, 6.9% for IgG2, 2.53% for IgG3, and 8.2% for IgG4.

Anti-CGL subclass activities in healthy blood donors. In an initial series of assays, we looked for anti-CGL IgG subclass activities in serum samples from 150 healthy blood donors of whom 52 (35%) were found by ELISA to be IgG positive toward the CGL antigen (10). All 52 IgG-positive samples with endpoint activities varying from 1:200 to 1:6,400 had anti-CGL IgG2. One individual considered to be IgG nega-

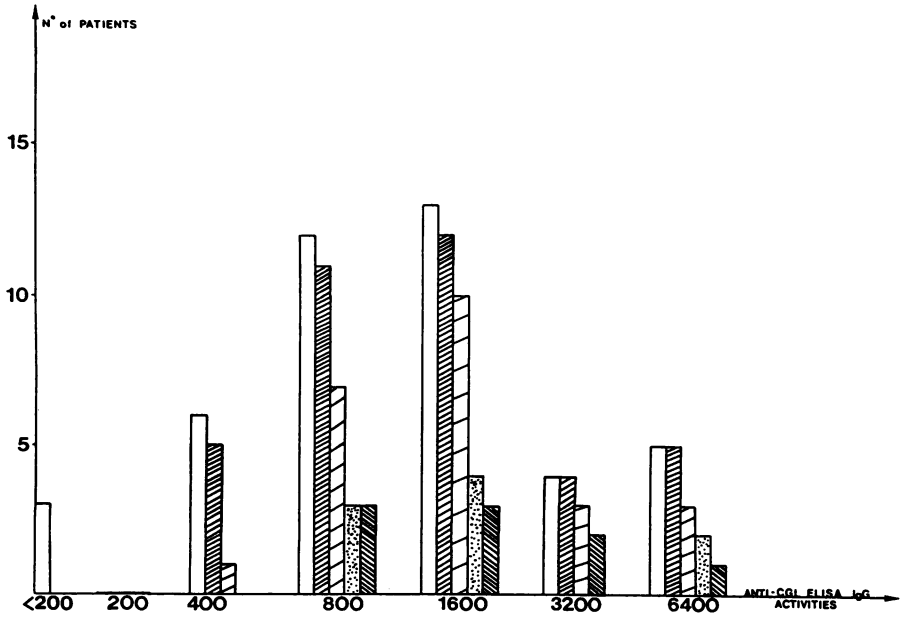

FIG. 3. Distribution of anti-CGL total and subclass IgG activities in the same population of patients for which data are presented in Fig. 2. Data are for patients during their hospitalization. The ordinate, the abscissa, and the symbols are as described in the legend to Fig. 2.

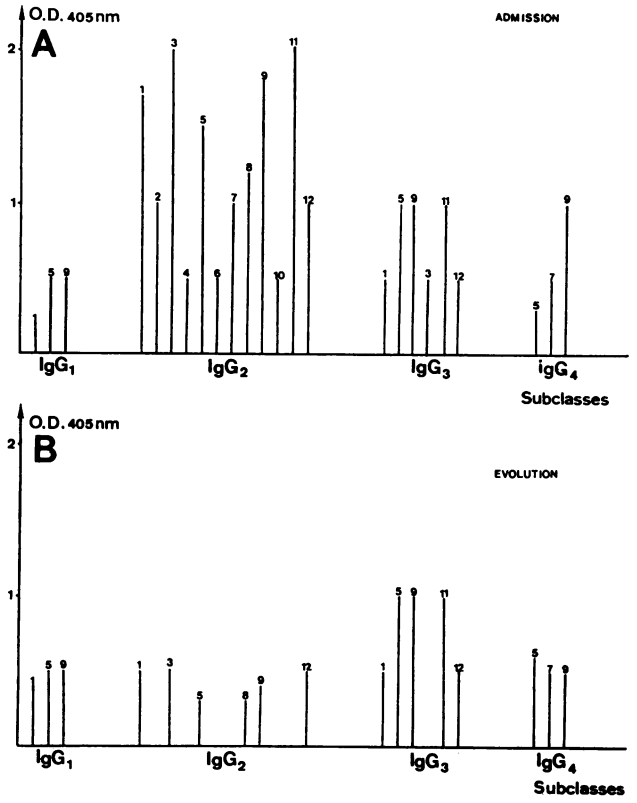

FIG. 4. Activities of the anti-CGL subclass in the 12 patients who died, on admission (A) and just before death (B). Ordinate, ODs at 405 nm; abscissa, IgG subclass types. Each vertical line represents one of the patients of this group, represented by a number from 1 to 12.

tive in the ELISA for IgG displayed a slight IgG1 activity in the subclass-specific assay. This fact suggests that in some cases the subclass assays are somehow more sensitive than the ELISA for whole IgG. We explained this by considering the starting dilutions of the serum samples in both assays.

Twenty-nine individuals were found to be IgG1 positive, and 19 had IgG3 activity. In nine individuals, we detected IgG1, IgG2, and IgG3 activities. None of the individuals had IgG4 activity, and we generally observed that IgG3 activities, if any, were relatively low with regard to IgG2 and IgG1, which were revealed to be predominant and to occur the most frequently in this group.

On the other hand, no differences in the distribution of the subclass activities were observed between males and females. Results of a representative series of experiments with sera from 20 individuals who were investigated for their subclass activities are given in Table 1.

Anti-CGL IgG subclass activities in patients displaying an acute gram-negative infection. The distribution of anti-CGL IgG activities determined by ELISA and subclass activities in patients on admission and during their hospitalization are reported in Tables 2 and 3, respectively. We followed 43 patients who presented clinical and bacteriological signs of acute gram-negative infection during their hospitalization. In this group, 41 were found by ELISA to be anti-CGL IgG positive on admission. All had IgG2, 4 had IgG1, 18 had IgG3, and 5 had IgG4. Of the 41 patients, 2 had IgG1, IgG2, and IgG3 and 1 had all four subclasses. The two IgG-negative patients became IgG positive after at least 2 weeks. Thirty patients displayed at least a fourfold rise of their whole IgG activity (Table 3). Only one of them died, which was from heart failure. The remaining 13 patients died of an infectious episode, leading to irreversible septic shock or organ failure. In 1 patient, we did not observe any IgG increase, and a significant decrease was demonstrated in the other 12 patients.

When the data in Tables 2 and 3 are compared, it appears that from the 30 patients with either a significant increase in IgG or an IgG seroconversion detected by ELISA, 5 acquired IgG1, 6 acquired IgG3, and 4 acquired IgG4.

Generally, in the group of patients IgG1 and IgG4 activities were found at relatively low levels, when compared with IgG2 and IgG3 activities, and were found at very low frequencies.

All patients displaying IgG4 activities had anti-CGL IgG activities of $\geq$1:800 as determined by ELISA on admission and during serological evolution. On the other hand, they all had positive blood cultures. These facts suggest that the development of an anti-CGL IgG4 activity may be considered as an indicator of active response toward a severe infection. However, three patients displayed a bacteremia without developing IgG4.

In addition we observed (Fig. 2 and 3) that patients with a significant rise in total IgG activities also displayed an increased occurrence of IgG2 activities, as well as the apparition of IgG3 in 6 of the 30 patients. Moreover, IgG2 levels increased in all individuals (data not shown). This observation indicates that IgG2 and IgG3 are the main factors that determine the endpoint activity of the total anti-CGL IgG, as determined by ELISA, the activities of IgG1 and IgG4 remaining relatively weak. Conversely, in 12 of the 13 patients who died, we observed a striking drop in anti-IgG2 activity, whereas activities of most of the other subclasses remained stable, with the exception of that in 1 patient, who lost IgG3 activity (Fig. 4).

DISCUSSION

In this study we developed an ELISA for the detection of anti-CGL IgG subclass antibodies with the goal to improve the anti-CGL IgG ELISA that we had previously set up to carry out a study (10) of antibodies to enterobacterial Re glycolipid and lipid A.

Lowe et al. (8) observed a low cross-reactivity in the system that they used due to the presence of contaminants in the purified human immunoglobulins used for such specificity controls. Therefore, the monoclonal antibodies were evaluated for their efficiency in an indirect double antibody sandwich ELISA in which the first marker antibody was the subclass-specific mouse monoclonal antibody. All four monoclonal antibodies detected their related IgG subclass without any significant cross-reactivities.

Our estimations of IgG subclass-specific activities should be considered qualitative and semiquantitative rather than quantitative. Therefore, we considered it to be more convenient, to simplify the expression of results, to express the activities as ODs.

In the healthy subjects, the IgG2 subclass was detected in all IgG-positive samples. IgG1 was found at a higher frequency in healthy blood donors than in patients on admission. Anti-CGL IgG3 in the blood donors was found more occasionally, and we never detected IgG4 in this group.

Our major observations concern (i) the predominant role of the IgG2 subclass in healthy individuals and in patients with an active serological response toward gram-negative

bacteria, (ii) the presence or the apparition of IgG4 only in our patients with confirmed bacteremia, and (iii) the direct correlation between the abatement of IgG2 activity and the fatal evolution of the illness.

Siber et al. (15) have demonstrated a correlation between IgG2 concentrations in serum and the antibody response to bacterial polysaccharide antigens. According to these authors, the IgG2 concentration can be considered as a marker for predicting the ability of the subject to elicit an antibody response toward polysaccharides. However, in children the IgG2 level is not a good predictor of antibody response to polyvalent pneumococcal vaccine (16). Different explanations can be postulated to explain the discrepancy between the latter findings and those of Siber et al. (15), such as a gradual shift with age in children from IgG1 to IgG2 in their responses to polysaccharide antigen. Yount et al. (19) have shown that the antibody response directed against several carbohydrate antigens, including dextran, levan, and teichoic acid, is attributed primarily to IgG2. It can be speculated that antibodies to pneumococcal and *H. influenzae* type b polysaccharides are also predominantly of the IgG2 subclass. Indeed, Oxelius (11) has reported that in a family with recurrent bronchopulmonary infections most frequently due to *H. influenzae*, IgG2 antibodies remained undetectable. In addition, Schur et al. (13) have described three patients with lifelong susceptibilities to pyogenic infections that induced a progressive pulmonary disease. These patients had normal or nearly normal total immunoglobulin levels but demonstrated selective deficiencies of IgG1, IgG2, or another subclass. From these points of view, the occurrence of low IgG2 levels in these patients may lead to the conclusion that recurrent infections are due to a deficiency in specific antibodies to bacterial polysaccharide antigens.

In our population of patients, we predominantly detected anti-CGL activities due to the IgG2 and IgG3 subclasses. On the contrary, in the blood donor group, the IgG1 activities were found at a higher frequency, with more elevated relative activity. The presence of IgG4 antibodies can be considered as a marker of severe infection, since it occurs only in bacteremic patients with an anti-CGL antigen activity of $\geq$1:800.

Our observation concerning the decrease of anti-CGL IgG2 before the death of patients undergoing a septic shock or irreversible organ failure due to infection also suggests that the anti-CGL IgG2 monitoring could be used as a marker of the evolution of illness in this group of patients. The dramatic drop of anti-CGL IgG2 activity during a short period may indicate that the reaction of anti-CGL with bacterial polysaccharide at high concentrations contributes to the absorption of specific IgG2, giving rise to a loss of efficiency of the immune antibody response.

Finally, we suggest that a seroprophylaxis with immunoglobulin concentrated from pooled plasma of healthy donors or convalescent patients with high IgG2 activities could offer an interesting approach for the preventive management of patients at high risk of gram-negative bacterial infections that constitute an important limiting factor to survival in these particular groups.

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