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Association with Phagocytic Inhibition of Anti-*Pseudomonas aeruginosa* Immunoglobulin G Antibody Subclass Levels in Serum from Patients with Cystic Fibrosis

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Serum from cystic fibrosis patients colonized with *Pseudomonas aeruginosa* specifically inhibits phagocytosis of *P. aeruginosa* by alveolar macrophages. Serum was examined for *P. aeruginosa* lipopolysaccharide-specific immunoglobulin G (IgG) subclass levels (by enzyme-linked immunosorbent assay) and for the effect on macrophage phagocytosis (by radiolabeled *P. aeruginosa* uptake). Sera from cystic fibrosis patients with no known *P. aeruginosa* colonization history had negligible amounts of lipopolysaccharide-specific IgG and a mean phagocytic enhancement of 5%. The sera of normal volunteers also had negligible amounts of lipopolysaccharide-specific IgG. Serum from cystic fibrosis patients with *P. aeruginosa* respiratory tract infections had substantial titers (range, 1:20 to 1:1,280) of lipopolysaccharide-specific IgG2, IgG3, and IgG4 and a mean phagocytic inhibition of 56%. However, these patients had low or absent titers of lipopolysaccharide-specific IgG1. No consistent variation in the level of individual IgG subclasses in the sera of colonized patients was observed, as determined by radial immunodiffusion. The results suggest that during *P. aeruginosa* infection phagocytosis-inhibitory activity develops coincident with production of lipopolysaccharide-specific IgG subclasses.

A common feature of cystic fibrosis (CF) is the chronic *Pseudomonas aeruginosa* pulmonary infection which contributes to the morbidity and mortality of these patients (20). The confinement of this infection to the lungs suggests a local immune response defect (18). Serum from CF patients with chronic *P. aeruginosa* pulmonary infection inhibited the in vitro phagocytosis of *P. aeruginosa* by both normal (15, 18) and CF alveolar macrophages (18). However, CF macrophage phagocytosis was unimpaired in the presence of normal serum (18). When CF and normal sera were mixed together in the phagocytic assay, the inhibition remained (15). This suggested a blocking activity rather than a deficiency in CF serum. The inhibition was specific for *P. aeruginosa* since phagocytosis of *Serratia marcescens* and *Staphylococcus aureus* was unimpaired (15). The observed specificity suggested that inhibition was antibody mediated. Receptors for immunoglobulin M (IgM) have not been demonstrated on human alveolar macrophages (7). Although IgG receptors are known to be present on human alveolar macrophages (7), IgG subclass-specific receptors have not been described. However, IgG1 and IgG3 have been shown to bind strongly to human monocytes, whereas IgG2 and IgG4 bind weakly (1, 3). Thus, it is possible that nonopsonic IgG subclass antibodies, directed to *P. aeruginosa* antigens, could interfere with macrophage phagocytosis. Fick et al. (2) have demonstrated that *P. aeruginosa* lipopolysaccharide (LPS)-specific IgG obtained from the serum of chronically infected CF patients inhibits the ability of alveolar macrophages to phagocytize *P. aeruginosa*.

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Previous work has shown that cats immunized with *P. aeruginosa* LPS develop phagocytosis-inhibitory activity in their sera (12). This observation also suggested that chronic LPS exposure, resulting from *P. aeruginosa* infection, might be important in the development of phagocytosis-inhibitory activity in the serum of CF patients. The present study provides evidence that as a result of chronic pulmonary infection with *P. aeruginosa* CF patients produce nonopsonic IgG subclasses directed to LPS, concurrent with the development of phagocytosis-inhibitory activity.

MATERIALS AND METHODS

Selection of patients. CF patients of both sexes were selected based on their *P. aeruginosa* culture history. The noncolonized group consisted of 10 CF patients, ranging in age from 1 to 26 years, who had never had *P. aeruginosa* recovered from a respiratory tract culture. The colonized group consisted of 10 CF patients who had had *P. aeruginosa* isolated from respiratory tract cultures consistently for several years. These patients were from 10 to 31 years old. Eleven normal volunteers (adult) ranging from 15 to 26 years and 12 normal children ranging from 1 to 14 years were also studied.

Collection of serum samples. Blood from both CF patients and normal volunteers was collected and clotted at room temperature. Serum was aliquotted and stored at −70°C until used.

Radial immunodiffusion. IgG subclass kits (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) were used. Results were compared with normal total IgG and IgG subclass ranges given in the literature accompanying the kits.

Enzyme-linked immunosorbent assay. An enzyme-linked immunosorbent assay was used to determine IgG subclass

TABLE 1. IgG subclass levels in sera of *P. aeruginosa*-colonized and noncolonized CF patients

Patient no.	Age (yr)	IgG level (mg/dl)[a]				
		IgG1	IgG2	IgG3	IgG4	Total
Colonized						
151	18	1,100	200	132[b]	13	1,445
897	13	880	320	117	60	1,377
154	11	950	460	151	119	1,680[b]
883	12	300[c]	160	65	24	549
270	10	800	260	17[c]	29	1,106
375	11	1,125[b]	340	87	47	1,599
644	14	650	400	72	65	1,187
816	16	1,085	200	60	73	1,418
24	16	850	150	88	18	1,106
Noncolonized						
110	7	806	104[c]	53	9	972
309	12	1,399[b]	248	75	133	1,855[b]
768	14	981	296	80	104[b]	1,461
725	7	478	117	42	26	633
801	1	697[b]	49	42	4	792
862	4	497	187	49	27	760
910	12	891	202	109	118	1,320
1019	4	1,041[b]	232	148[b]	61	1,482
1028	26	497	49[c]	36[c]	5	587
1017	5	760	63[c]	39	10	872
Normal volunteers (n = 11)[d]	20.0 ± 3.5 (15–26)	800 ± 200 (534–1,201)	284 ± 105 (117–435)	56 ± 16 (21–86)	39 ± 32 (10–107)	1,191 ± 258 (872–1,589)

[a] Determined by radial immunodiffusion. No serum from patient 59 was available for assay.
[b] Value is above the range of IgG subclass levels for age-matched normal individuals.
[c] Value is below the range of IgG subclass levels for age-matched normal individuals.
[d] Data for normal volunteers show means ± standard deviations, with ranges within parentheses.

titers specific for *P. aeruginosa* LPS international serotype 6. LPS was extracted by the trichloroacetic acid method (14) from strain 1369 (provided by P. V. Liu, University of Louisville Health Sciences Center, Louisville, Ky.). The lyophilized LPS preparation was stored at 4°C until used. Multiwell microassay plates (Falcon Microtest III; Becton Dickinson Labware, Oxnard, Calif.) were coated with the LPS antigen (1 μg/ml) in 20 mM phosphate-buffered saline (PBS), pH 7.2, and incubated at 4°C overnight. The plates were washed three times with PBS–0.05% Tween 20. After washing, 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was added, and the plates were incubated at 37°C for 30 min and then rinsed again. Serum samples were diluted to 1,000 mg of IgG per dl with PBS-Tween (unless the initial IgG concentration was below that amount) to facilitate comparison between samples. Twofold serial dilutions of serum in PBS-Tween were added to four groups of duplicate wells, incubated at 37°C, and washed with PBS-Tween. Mouse anti-human IgG1 (clone JL512; GIBCO Laboratories, Grand Island, N.Y.), IgG2 (clone GOM-1), IgG3 (clone SJ-33), and IgG4 (clone SK-44; Miles) monoclonal antibodies were added individually to the four groups of wells and incubated at 37°C. All products were tested and certified for subclass monospecificity and lack of cross-reactivity by the manufacturers. The plates were washed with PBS-Tween, and affinity-purified, peroxidase-conjugated goat anti-mouse IgG (Pel-Freez, Rogers, Alaska) was added. After an incubation and wash, a substrate solution of *o*-phenylenediamine hydrochloride dissolved in 0.05 M Na_2HPO_3–0.025 M citrate buffer (pH 5.0), with 0.2 μl of 30% H_2O_2 per ml of buffer, was added to each well and incubated at room temperature in the dark. The reaction was stopped by the addition of 5 N H_2SO_4. The absorbance at 492 nm of each well was measured with an MR 600 Microplate Reader (Dynatech Laboratories, Inc., Alexandria, Va.). The endpoint for each subclass was expressed as the reciprocal of the dilution of serum whose absorbance was at least 0.150 units above that of background wells lacking the LPS antigen. For some experiments, tetanus and diphtheria toxoids (Lederle Laboratories, Pearl River, N.Y.) were used as control antigens.

Phagocytic assay. Human alveolar macrophages were obtained by fiberoptic bronchoscopy from normal volunteers (15). The cells were washed, allowed to adhere to glass cover slips, and cultured in McCoy 5A medium for 24 h at 37°C in a humidified 5% CO_2 atmosphere. P3, a serotype 6 strain of *P. aeruginosa*, was labeled with [^{3}H]leucine during log phase growth in Koser citrate at 37°C (19). The phagocytic assay was performed as previously reported (17). Briefly, quadruplicate cover slips were incubated for 20 min at 37°C in the presence of radiolabeled bacteria in CF or normal serum. One of the quadruplicate cover slips was stained with Diff-Quik stain (American Scientific Products, McGaw Park, Il.) to ascertain that bacteria were cell associated. The remaining three cover slips were used to assess the amount of phagocytosis by determining the radioactivity. The percent inhibition of phagocytosis was calculated as follows: {1 − [(^{3}H bacterial uptake in CF serum)/(^{3}H bacterial uptake in normal serum)]} × 100. Data expressed as percent inhibition (or enhancement) allowed a comparison to be made between different assays.

Serotyping. *P. aeruginosa* cultures of various morphologies from chronically infected CF patients were serotyped as described elsewhere (16) with *P. aeruginosa* antisera (International Serotyping Scheme; Difco Laboratories, Detroit, Mich.). Examination of cultures from a patient was limited to samples taken before the date of their serum collection.

Statistics. The *t*-test for independent, unequal variances was used for comparing phagocytic inhibition values for the various groups (4).

RESULTS

Serum samples from CF patients colonized with *P. aeruginosa* and those not colonized (as determined by culture history) were compared. For both groups, the total IgG concentration was determined. In addition, specific subclass antibodies to *P. aeruginosa* LPS and the level of phagocytosis of *P. aeruginosa* by normal alveolar macrophages in the presence of the same serum samples was evaluated. In this way, the IgG subclasses directed to LPS and the effect of the serum on phagocytosis could be related.

Total IgG and IgG subclass concentrations. The concentration of each IgG subclass in the serum of colonized and noncolonized patients was determined by radial immunodiffusion (Table 1). The results indicate that all nine colonized patients tested had concentrations of IgG2 and IgG4 within the age-matched normal range. Only two of nine patients, 883 and 375, had IgG1 concentrations below and above normal range values, respectively; the remainder had IgG1 levels within the normal range. Similarly, two patients, 151 and 270, had IgG3 levels above and below normal range levels, respectively, whereas the rest had IgG3 concentrations within normal range values. In the noncolonized group, 3 of 10 patients (309, 801, and 1019) had IgG1 subclass levels above the normal range; 3 patients (110, 1028, and 1017) had IgG2 levels below the normal range; patient 1019 had an IgG3 level above the normal range and patient 1028 had an IgG3 level below the normal range; one patient (768) had an IgG4 level above the normal range. Only one patient (154) in the colonized group and one patient (309) in the noncolonized group had a total IgG concentration above the range of normal serum IgG values. For comparison with patient sera, additional sera from 11 normal adults (ages 15 to 26 years) and 12 children (ages 1 to 14 years) were examined by radial immunodiffusion. For the adult population, only 1 subclass determination (IgG3) of 44 was below the normal range; the total IgG concentration for all 11 individuals was within the normal range. Slightly more variability was seen in the pediatric group, where 5 of 48 determinations were outside the normal range; however, the total IgG level was normal for all children tested.

LPS-specific IgG subclass titers. Titers of 1:20 or greater for IgG1 were present in only 2 of 10 colonized patients (Table 2). All patients had antibodies to LPS type 6 in IgG subclasses 2, 3, and 4. Serotyping showed that four of seven patients for whom isolates were available from before the serum collection date had documented colonization with *P. aeruginosa* serotype 6.

TABLE 2. CF patients with *P. aeruginosa* colonization

Patient no.	Years colonized[a]	LPS-specific IgG subclass titer[b]				% Inhibition of phagocytosis
		IgG1	IgG2	IgG3	IgG4	
151	5	—	640	40	160	52
897	4	—	160	640	80	56
154	10	—	640	20	80	55
883	6	—	160	1,280	320	62
270	4	—	320	640	160	53
375	11	—	1,280	160	160	50
59	2	160	320	160	160	51
644	2	—	1,280	20	160	61
816	13	20	160	2,560	160	59
24	16	—	640	160	160	57

[a] Length of documented colonization at Rainbow Babies and Childrens Hospital.
[b] Dashes (—) indicate titers of <20. Values are reciprocals of endpoint dilutions.

TABLE 3. CF patients without *P. aeruginosa* colonization

Patient no.	LPS-specific subclass titer[a]		% Inhibition of phagocytosis[b]
	IgG 3	IgG 4	
110	—	—	6
309	—	—	0
768	—	40	21
725	—	20	−22
801	—	—	5
862	—	—	−35
910	—	40	12
1019	20	160	2
1028	—	—	−51
1017	—	—	14

[a] Dashes (—) indicate titers of <20. Values are reciprocals of endpoint dilutions.
[b] Enhancement of phagocytosis is indicated by a negative number.

The possibility that the monoclonal antibody used could not detect IgG1 bound to LPS was examined. Since most individuals have been vaccinated against diphtheria and tetanus, diphtheria and tetanus toxoids were used as antigens in the same ELISA procedure. Five of five serum samples from normal volunteers had IgG1 antibodies to the toxoids (titer, ≥1:20), thus confirming the functionality of the monoclonal anti-IgG1.

CF patients without *P. aeruginosa* infection had essentially no IgG antibodies specific for LPS type 6 (Table 3). Only 4 of 10 patients tested had IgG4 titers; only one of these four also had an IgG3 titer of 1:20. No patient had detectable levels of IgG1 or IgG2 (data not shown). Statistical analysis with the Mann-Whitney test was used for comparisons of the log titer between groups. Subclass levels for IgG2, IgG3, and IgG4 for colonized and noncolonized patients were statistically different ($P < 0.001$). IgG1 was not different between the two groups. These results were similar to those obtained with sera from five normal individuals who had no LPS type 6-specific IgG antibodies of any subclass.

Phagocytosis. Sera from the chronically infected patients (Table 2) all showed phagocytosis inhibition of 50% or greater with a mean of 56 ± 4%. This was highly significant in comparison to the noncolonized and normal groups ($P < 0.005$).

Sera from the noncolonized CF patients showed little inhibition or an enhancement of macrophage phagocytosis of *P. aeruginosa* (Table 3). Mean phagocytosis in the presence of serum for noncolonized patients was only slightly enhanced (−5 ± 23%). There was no significant difference in phagocytosis between normal volunteer serum and noncolonized patient serum.

DISCUSSION

The absence of an IgG1 response to LPS in most of the colonized CF patients was striking because this subclass comprises the majority of total IgG in serum and is opsonic. Antibody responses elicited in humans are often restricted to specific IgG subclasses for certain antigens. Human antibodies to dextrans, levans, and teichoic acid (21) as well as bacterial polysaccharides (13) are primarily IgG2. Carbohydrates from group A streptococci stimulate the production of

IgG2 and IgG3 (8). *P. aeruginosa*-colonized CF patients have been reported to have elevated levels of IgG4, although the antigen(s) to which the IgG4 was directed was not defined (5).

Although there were elevated titers of IgG subclasses 2, 3, and 4 directed to LPS in the serum of the colonized patients, the individual IgG subclass concentrations were predominantly within normal subclass ranges for pediatric and adult populations as reported by Oxelius (6). Among serum samples from the colonized patient group, only 4 of 36 subclass levels were outside the normal range. The levels of IgG4 in the colonized CF patients were comparable to those concentrations reported by Oxelius (6) and Schur et al. (9) for age-matched normal individuals. In contrast, Shakib et al. (10, 11) reported elevated IgG4 levels in 7 of 16 CF patients, but the baseline levels of IgG4 in his normal population were lower than those of Oxelius (6) and Schur et al. (9). As a result, the significance of the reported elevated IgG4 concentrations in colonized CF patients is unclear. From our study, it appears that in the sera of *P. aeruginosa*-colonized patients, no single subclass is consistently elevated or depressed in spite of antibody responses against *P. aeruginosa* LPS. In serum samples from noncolonized patients, 9 of 40 subclass determinations were outside the normal range. However, no consistent pattern in the deviations could be detected, and some normal individuals also had subclass levels outside the normal range.

The total IgG concentration in the serum of the CF patients was determined by radial immunodiffusion and compared with age-matched normal values. Only two patients had elevated total IgG, one patient in the colonized group and one in the noncolonized group.

Sera with high LPS-specific IgG2, IgG3, and IgG4 titers also contained phagocytosis-inhibitory activity. Fick et al. (2) proposed that the phagocytosis-inhibitory activity of CF sera is due to a defect in the Fc portion of *P. aeruginosa*-specific IgG, and this prevents proper receptor attachment and internalization by macrophages. However, these same investigators also postulated that an alteration in the normal ratio of IgG subclasses may affect phagocytosis. The necessary ratio of opsonic to nonopsonic subclasses for phagocytic enhancement or inhibition is unknown, and further work with combinations of individual subclasses will be required to explore this fully. Other workers have proposed that enhanced titers of IgG4 directed to *P. aeruginosa* antigens are responsible for the phagocytic inhibition (5). Our results suggest that the presence of nonopsonic IgG2 and IgG4 directed to LPS may decrease the effectiveness of macrophage phagocytosis of *P. aeruginosa*. It is possible that these nonopsonic antibodies compete with opsonic IgG3 (since IgG1 levels versus LPS are low or absent), thus coating the bacteria with enough nonopsonic antibody to inhibit phagocytosis.

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