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Immunoglobulin G Subclass Response of Juvenile Periodontitis Subjects to Principal Outer Membrane Proteins of *Actinobacillus actinomycetemcomitans*

MARK E. WILSON[1]* AND ROBERT G. HAMILTON[2]

Department of Oral Biology, School of Dental Medicine, State University of New York at Buffalo, Buffalo, New York 14214,[1] and Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224[2]

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The cell envelope of *Actinobacillus actinomycetemcomitans* includes a number of outer membrane proteins (OMPs) which appear to be important targets for immunoglobulin G (IgG) antibodies in sera from localized juvenile periodontitis (LJP) patients. In this study, we examined the subclass distribution of IgG antibodies reactive to the 16.6- and 29-kDa OMPs of *A. actinomycetemcomitans* in sera from LJP patients and periodontally healthy individuals. Antibody responses were determined in a quantitative enzyme-linked immunosorbent assay that employed human IgG subclass-restricted monoclonal antibodies. High-titer LJP sera (93% black; geometric mean titer, 32,673) were found to contain significantly elevated levels of IgG1, IgG2, and IgG3 antibodies to the 29-kDa OMP of *A. actinomycetemcomitans*, compared with those of low-titer LJP sera (mean titer, 1,421) and sera from periodontally healthy, race-matched control subjects. The concentration of IgG2 antibody to this protein was greater than or equal to the corresponding IgG1 concentration in 7 of 14 high-titer sera, although mean IgG1 and IgG2 concentrations were not significantly different. The concentrations of IgG1 and IgG2 antibodies to the 16.6-kDa protein were also significantly elevated in LJP sera, although of considerably lesser magnitude than that observed for the 29-kDa protein. The IgG2 response to the 29-kDa protein could not be attributed to the presence of IgG2 antibodies to lipopolysaccharide contaminants or to Fc-binding activity, nor does this molecule appear to be a glycoprotein. Hence, LJP subjects produce IgG2 antibodies, as well as IgG1 and IgG3 antibodies, directed to at least one of the major OMPs of *A. actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans is a capnophilic gram-negative coccobacillus which has been implicated in the etiology of localized juvenile periodontitis (LJP) (38). LJP patients often exhibit markedly increased serum immunoglobulin G (IgG) antibody titers to *A. actinomycetemcomitans* antigens (6). Previous studies have provided evidence to support the hypothesis that the antibody response to *A. actinomycetemcomitans* is protective (10, 26). However, information regarding the key mechanism(s) of antibody-mediated host defense against this organism is limited. LJP sera have been found to contain IgG antibodies reactive to a number of *A. actinomycetemcomitans* antigens, including (i) serotype-defining lipopolysaccharide (LPS) (4, 7, 37), (ii) lktA (a member of the RTX family of toxins) (30), and (iii) outer membrane proteins (OMPs) (31, 32). The relative importance of these antigens in eliciting the production of IgG antibodies capable of hastening the elimination of *A. actinomycetemcomitans* has not been defined.

Serum IgG consists of four distinct subclasses, which differ in their respective serum concentrations and certain key biologic properties (28). Moreover, the subclass distribution of IgG antibodies produced as a consequence of either natural infection or vaccination can vary, depending upon the nature of the eliciting antigen(s). Typically, protein antigens induce mainly IgG1 antibodies, with smaller amounts of IgG3 and IgG4 antibodies produced (14, 15). On the other hand, IgG antibodies reactive to carbohydrate antigens, including LPS, are commonly restricted to the IgG1 and IgG2 subclasses, with the latter often predominating. Antibodies of the IgG1 and IgG3 subclasses have been found to exhibit superior complement-fixing activities to those of the IgG2 subclass. IgG4 antibodies lack detectable complement-fixing activities (28). It has also been observed that IgG1 and IgG3 antibodies interact more effectively with phagocyte Fcγ receptors than do either IgG2 or IgG4 antibodies, although an allotypic form of the low-affinity IgG receptor (CD32) has recently been reported to recognize IgG2 efficiently (25).

Given that the IgG subclass(es) produced as a consequence of bacterial infection may influence the extent to which these antibodies contribute to host defense, a number of groups have begun to evaluate the IgG subclass response of LJP subjects to *A. actinomycetemcomitans*. Employing a sonicate antigen preparation of *A. actinomycetemcomitans* Y4, Ling and coworkers demonstrated that among high-titer LJP sera, IgG antibodies to this organism were predominantly of the IgG2 subclass (18). We demonstrated that LJP sera contain markedly elevated levels of IgG antibodies directed to serospecific determinants in LPS from *A. actinomycetemcomitans* and that most of these antibodies belong to the IgG2 subclass (36). These observations have recently been confirmed by Lu and coworkers (19).

Although available evidence suggests that LPS may be the immunodominant antigen of *A. actinomycetemcomitans*, it is clear that IgG antibodies in sera from LJP patients also recognize other surface-accessible constituents of the outer membrane of this organism, notably OMPs (31). One such protein is a 29-kDa, heat-modifiable species which exhibits N-terminal sequence homology to the OmpA protein of *Escherichia coli* (32, 33). However, a 16.5-kDa protein has also been reported to be a key surface antigen of *A. actinomycetemcomitans* (3). In this study, we utilized a quantitative enzyme-linked immunosorbent assay (ELISA) to characterize LJP sera with respect

* Corresponding author. Mailing address: Department of Oral Biology, School of Dental Medicine, State University of New York at Buffalo, 3435 Main St., Foster Hall, Buffalo, NY 14214-3092. Phone: (716) 829-2590. Fax: (716) 829-3942.

TABLE 1. Concentrations of IgG subclass antibodies to the 29-kDa OMP in sera from LJP patients and periodontally healthy race-matched controls

Patient group	IgG subclass antibody concn[a] (range)			
	IgG1	IgG2	IgG3	IgG4
High-titer LJP (n = 14)	23.17[b] (2.31–95.66)	29.09[b] (6.90–133.84)	6.61[b] (0.10–22.75)	0.02 (0.001–16.02)
Low-titer LJP (n = 9)	3.12 (0.54–28.41)	0.53 (0.05–13.29)	ND[c]	ND
PH[d] (n = 5)	0.85 (0.11–3.27)	1.10 (0.13–4.48)	0.13 (0.10–0.20)	0.01 (0.003–0.085)

[a] Geometric mean concentration in micrograms per milliliter.
[b] Significantly different from mean IgG subclass concentration(s) of low-titer LJP and/or periodontally healthy groups ($P < 0.01$) by Wilcoxon-Mann-Whitney test.
[c] ND, not determined.
[d] PH, periodontally healthy, race-matched control subjects.

to the distribution of IgG subclass antibodies to these two OMPs of *A. actinomycetemcomitans*.

MATERIALS AND METHODS

Patient sera. Sera were obtained from 23 patients with LJP (mean age, 18.4 years; range, 10 to 31 years; 19 were black, 3 were white, and 1 was Asian) who received treatment at the Periodontal Disease Clinical Research Center, State University of New York at Buffalo. Diagnosis of LJP was made according to criteria described previously (37). Reciprocal IgG antibody titers to the 29-kDa OMP of *A. actinomycetemcomitans* in these sera were determined by ELISA (32). On the basis of this analysis, sera were subdivided into two groups, high-titer LJP (geometric mean IgG titer, 32,673; n = 14) and low-titer LJP (geometric mean IgG titer, 1,421; n = 9). Thirteen of the 14 high-titer LJP subjects were black, whereas the low-titer LJP group included 6 blacks, 2 whites, and 1 Asian. Sera from periodontally healthy black individuals were generously provided by H. Schenkein, Medical College of Virginia, Richmond, Va. Informed consent was obtained from all subjects that participated in this study.

Isolation of the 29-kDa OMP. *A. actinomycetemcomitans* Y4 (ATCC 43718) was maintained on 5% sheep blood agar supplemented with 1 g of yeast extract (Difco Laboratories, Detroit, Mich.) per liter, 1 mg of 3-phytylmenadione per liter, and 5 mg of equine hemin III (Sigma Chemical Co., St. Louis, Mo.) per liter and incubated at 37°C in humidified 5% CO_2. Broth cultures were prepared in NIH thioglycollate (Difco) supplemented with 1 g of sodium bicarbonate per liter. Organisms were incubated as described above and harvested in mid-logarithmic phase. The 29-kDa OMP was isolated as described previously (32). Briefly, a total membrane fraction was obtained by sonication and differential centrifugation, after which the inner cytoplasmic membrane was solubilized in 1% sodium lauroyl sarcosinate (Sigma). The 29-kDa protein was solubilized from an octylglucoside-sodium chloride-insoluble fraction of the outer membrane by incubation at ambient temperature in 20 mM sodium phosphate (pH 7.5) that contained 1% sodium dodecyl sulfate (SDS) and was further purified by hydroxylapatite chromatography.

Isolation of the 16.6-kDa OMP. The 16.6-kDa protein was isolated according to the procedure employed by Munson and Granoff to solubilize the P6 protein from *Haemophilus influenzae* (22). This protein is insoluble under the conditions of ambient temperature used to solubilize the 29-kDa protein but is soluble in SDS-containing buffer at elevated temperatures. Briefly, the insoluble pellet recovered after solubilization of the 29-kDa protein was resuspended in 0.1 M Tris–0.5 M NaCl (pH 8.0) that contained 1% SDS and 0.1% 2-mercaptoethanol and incubated at 60°C for 30 min. Following centrifugation at 100,000 × g for 1 h, the 16.6-kDa protein was recovered in the supernatant fraction. This material was further purified by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a Bio-Rad 491 prep cell (Bio-Rad, Richmond, Calif.). The 16.6-kDa-enriched fraction was solubilized at 50°C for 5 min in sample buffer that consisted of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.003% bromophenol blue in 0.063 M Tris HCl (pH 6.8). The sample was applied to a prep cell column (28-mm internal diameter) with a 3% stacking gel (1.0 cm) and a 14% resolving gel (4.8 cm) and electrophoresed at 10 W of constant power. When the tracking dye emerged from the bottom of the preparative gel, 2-ml fractions were collected at an elution rate of 1 ml/min over 2 to 3 h. Aliquots of each fraction were incubated in sample buffer at 50°C and subjected to analytical SDS-PAGE with a vertical-slab (11 by 8 cm) electrophoresis cell (Hoefer Scientific Instruments, San Francisco, Calif.) as described previously (32). Fractions that contained the 16.6-kDa protein were pooled, concentrated, and dialyzed against phosphate-buffered saline. Protein concentrations were determined by A_{280}, with bovine serum albumin as a standard.

Evaluation of LPS contamination. Several approaches were utilized to assess the extent of LPS contamination in OMP preparations. First, endotoxin content was determined by *Limulus* lysate gelation assay with a Pyrotell kit (Associates of Cape Cod, Woods Hole, Mass.) and and found to be less than 0.6 ng per mg of 29-kDa protein and less than 15 ng per mg of 16.6-kDa protein. Secondly, LPS content was estimated on silver-stained SDS-polyacrylamide gels (22, 32) and found to be less than 0.4%. Thirdly, probing electroblotted proteins with LJP serum that contained elevated levels of IgG antibody reactive to purified *A. actinomycetemcomitans* LPS failed to reveal the presence of LPS.

IgG subclass ELISA. Serum IgG subclass antibodies reactive to the isolated 16.6- and 29-kDa OMPs of *A. actinomycetemcomitans* were measured by an ELISA that employed subclass-restricted murine monoclonal antibodies as described previously (36) except that the plates were coated with an optimal concentration of isolated OMPs in 0.015 M carbonate (pH 9.6). IgG subclass concentrations were determined by heterologous interpolation of a dose-response curve generated from each ELISA plate with chimeric human-mouse antibodies (Hybridoma Reagent Laboratory, Kingsville, Md.) which consisted of mouse variable regions with specificity for the hapten 4-hydroxy-3-nitrophenylacetyl and human constant regions for IgG1, IgG2, IgG3, or IgG4 (12, 36). In some experiments, affinity-purified IgG2 antibodies from LJP and pooled healthy human sera prepared as described elsewhere (35a) were employed in place of unfractionated sera.

Determination of total IgG and IgG subclass concentrations in patient sera. IgG subclass concentrations in sera were determined by means of immunoenzymetric assay (13). Total IgG concentrations in sera were determined by the summation of individual IgG subclass concentrations. These summated values were within 15% of total IgG concentrations as determined by independent analysis by a radial immunodiffusion assay (Sanofi Diagnostics Pasteur, Chaska, Minn.).

Competitive binding ELISA. As a further means by which to define the contribution of LPS contamination to the observed IgG subclass response to the isolated 29-kDa OMP, a series of competitive binding ELISAs were performed. Briefly, high-molecular-weight LPS was obtained from *A. actinomycetemcomitans* Y4 by extraction into hot aqueous phenol, gel filtration chromatography in LPS-disaggregating buffer, and nuclease digestion (37). A lipid-free oligosaccharide (LFO) fraction was prepared by incubating a 1% (wt/vol) suspension of LPS in 1% (vol/vol) acetic acid for 3 h at 100°C. The supernatant fraction was extracted with chloroform to eliminate residual lipid, desalted on Sephadex G-10, and lyophilized. This fraction contained only trace amounts of glucosamine and fatty acid and was thus designated LFO. Previous studies have demonstrated that IgG antibodies in LJP sera which are reactive to *A. actinomycetemcomitans* LPS are principally directed to the oligosaccharide moiety rather than to the lipid A region (37). Accordingly, the LFO fraction was employed in a competitive binding experiment designed to negate the contribution of LPS-reactive antibodies in ELISAs for quantifying IgG subclass response to the isolated 29-kDa OMP. LJP serum (10% [vol/vol]) was incubated for 1 h at 4°C in the presence of LFO (50 to 500 μg/ml) or buffer alone. Serum was serially diluted and transferred to enzyme immunoassay plates coated with either *A. actinomycetemcomitans* LPS or 29-kDa OMP, after which residual IgG subclass antibody concentrations were determined as described above.

Statistical analyses. Differences in mean concentrations of IgG subclass antibodies to the 16.6- and 29-kDa OMPs between LJP and periodontally healthy subjects were determined by the Wilcoxon-Mann-Whitney test. This test was also used to assess differences in total IgG and IgG subclass concentrations in sera among the various patient groups. Statistical differences in IgG subclass antibody concentrations among high-titer LJP subjects were determined by the Wilcoxon signed rank test.

RESULTS

IgG subclass response to the 29-kDa OMP. LJP sera often exhibit elevated titers of IgG antibodies reactive to the heat-modifiable OMP of *A. actinomycetemcomitans* (32). We sought to define the subclass distribution of IgG antibodies reactive to this OMP in high- and low-titer LJP sera as well as in sera from periodontally healthy, race-matched control subjects. As shown in Table 1, the geometric mean concentrations of IgG1, IgG2, and IgG3 antibodies to the 29-kDa OMP were signifi-

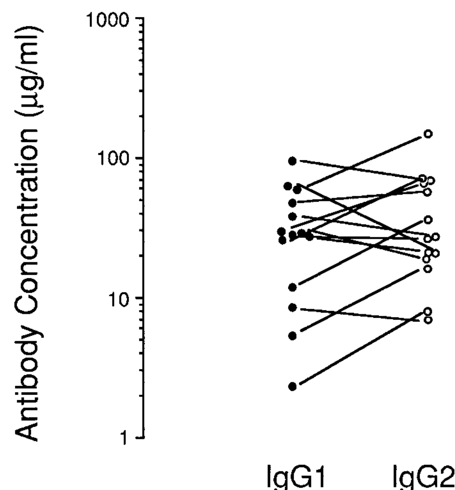

FIG. 1. Concentrations of IgG1 and IgG2 subclass antibodies to the 29-kDa OMP of *A. actinomycetemcomitans* Y4 in high-titer LJP sera.

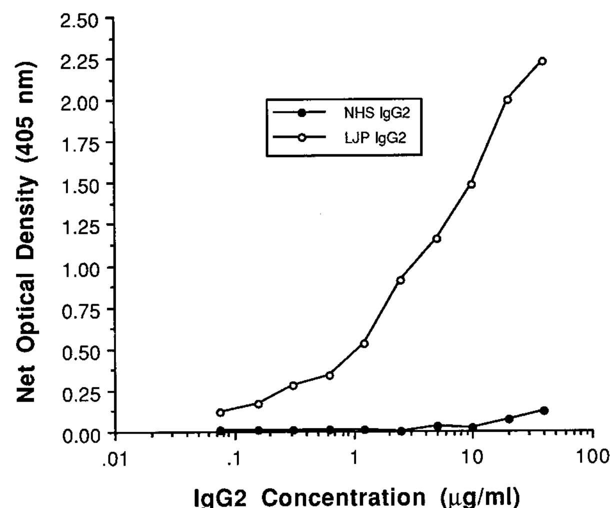

FIG. 2. Binding of affinity-purified IgG2 antibodies from high-titer LJP serum and pooled serum from periodontally healthy individuals to the isolated 29-kDa OMP of *A. actinomycetemcomitans*. NHS, pooled serum from periodontally healthy individuals.

cantly elevated in high-titer LJP sera, as compared with sera from low-titer LJP and periodontally healthy subjects. IgG4 subclass antibody concentrations in high-titer LJP sera were not significantly elevated. Geometric mean IgG1 and IgG2 antibody concentrations in low-titer LJP sera did not differ significantly from those of control sera, although there was a tendency toward an increase in the level of antibody activity for the IgG1 subclass.

Among the 14 high-titer LJP sera tested, 7 exhibited concentrations of IgG2 antibody to the 29-kDa OMP that were equal to or greater than the corresponding IgG1 concentrations (Fig. 1). In 6 of these 14 patients, the IgG2 antibody concentration was at least twofold greater than the IgG1 concentration. In contrast, in only 1 of 14 patients did the concentration of IgG1 antibody to the 29-kDa protein exceed the corresponding IgG2 concentration by twofold or more. The mean ratio of IgG2-to-IgG1 antibody concentration in this group was 1.58 ± 1.03. The geometric mean IgG2 concentration did not differ significantly from that of the corresponding mean IgG1 concentration, as determined by the Wilcoxon signed rank test, although both were significantly greater ($P <$ 0.001) than the mean IgG3 and IgG4 antibody concentrations in these sera.

Further evidence of specific binding of IgG2 antibodies in LJP serum to the 29-kDa OMP was derived from studies that employed affinity-purified IgG2 antibodies from LJP serum and pooled healthy human serum from periodontally healthy subjects. As depicted in Fig. 2, affinity-purified IgG2 antibodies obtained from the serum of a high-titer LJP subject exhibited strong reactivity to the 29-kDa protein. In contrast, affinity-purified IgG2 antibodies from pooled healthy human serum were only weakly reactive to this antigen. These results provide additional evidence that high-titer LJP sera contain IgG2 antibodies which specifically recognize the 29-kDa OMP of *A. actinomycetemcomitans*.

Competitive binding studies. We previously reported that LJP sera frequently contain markedly elevated levels of IgG antibodies which recognize *A. actinomycetemcomitans* LPS; most of these LPS-reactive antibodies belong to the IgG2 subclass (36). Many of the high-titer LJP sera employed in this study to define subclass response to the 29-kDa OMP also contained substantial amounts of IgG2 antibody to *A. actinomycetemcomitans* Y4 LPS. Several lines of evidence indicated that the isolated 29-kDa OMP employed in ELISAs contained LPS in only trace amounts. In view of the substantial amounts of IgG2 antibodies to the OMP present in high-titer LJP sera, we sought additional evidence that the OMP-reactive IgG2 antibodies were specific for this protein and not for residual LPS contaminants. Accordingly, we performed competitive binding studies designed to address this issue. LJP serum that contained elevated levels of IgG2 antibodies to the 29-kDa OMP, as well as antibodies to LPS prepared from the same strain, was preincubated with variable concentrations of an LFO fraction obtained by acetic acid hydrolysis of LPS. The effects of preincubation with LFO on IgG2 antibody response in serum to ELISA plates coated with either LPS or this OMP were subsequently determined. As shown in Fig. 3A, preincubation of serum with 50 to 250 μg of LFO per ml for 1 h resulted in a concentration-dependent decrease in IgG2 antibody response to LPS, with >90% inhibition seen at 250 μg of LFO per ml. In contrast, preincubation of LJP serum with LFO had a negligible effect on IgG2 antibody response to the 29-kDa OMP, even at concentrations twofold greater than those required to inhibit completely the IgG2 response to LPS (Fig. 3B). These results provide further evidence that LJP sera contain OMP-specific IgG2 antibodies which are distinct from those which recognize LPS-associated determinants.

Analysis of carbohydrate content of the 29-kDa protein. The presence of carbohydrate in the 29-kDa OMP preparation was assessed by several methods. First, this protein was unreactive in a glycan detection assay (Boehringer Mannheim, Indianapolis, Ind.) for glycoproteins. Secondly, when resolved on SDS-polyacrylamide gels, this protein was unreactive to staining with periodic acid-Schiff reagent. Finally, a 100-μg sample of the 29-kDa OMP was hydrolyzed in 2 N HCl for 6 h at 100°C and evaporated to dryness. Amino sugar content was determined by automated amino acid analysis on a Beckman 6300 system, with norleucine as an internal standard. The 29-kDa OMP was found to contain 15.32 μg of glucosamine and 11.24 μg of galactosamine per mg of protein, indicating that >97% of the OmpA molecule consists of protein.

Fc-binding properties of the isolated 29-kDa protein. It has recently been suggested that the outer membrane of *A. actinomycetemcomitans* possesses Fc-binding activity which may be

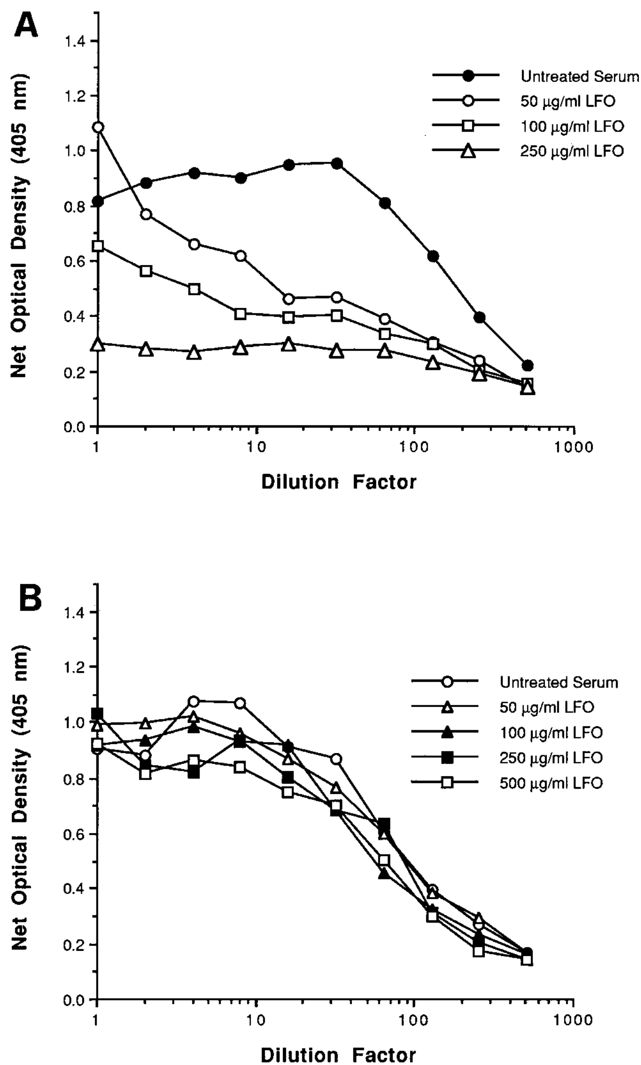

FIG. 3. Inhibition of IgG2 antibody response to *A. actinomycetemcomitans* LPS and the 29-kDa OMP by the LFO moiety of LPS. LJP serum that contained elevated levels of IgG2 antibodies to *A. actinomycetemcomitans* LPS and the 29-kDa OMP was preincubated for 1 h at 4°C in the presence of LFO (50 to 250 µg/ml) prepared by acetic acid hydrolysis of LPS (see Materials and Methods). Residual IgG2 antibody activity to either intact LPS (A) or isolated 29-kDa protein (B) was determined by ELISA.

ascribed to the 29-kDa OMP (21). This prompted us to examine the extent to which such Fc-binding activity might account for the binding of human IgG (particularly IgG2) antibodies in LJP sera to the purified 29-kDa protein. First, we compared the binding activities of intact polyclonal IgG and Fc fragments (prepared by papain digestion [9]) to the 29-kDa protein by employing either biotinylated anti-human IgG1 or IgG2 antibodies to assess the extent of binding. As shown in Fig. 4, intact IgG, but not Fc fragments, bound to the isolated 29-kDa protein. Similar results were obtained with either anti-human IgG2 (Fig. 4) or anti-human IgG1 (data not shown) to detect the binding of intact IgG or Fc fragments.

We subsequently assessed the capacity of human IgG Fc fragments to inhibit the binding of IgG antibodies in LJP serum to this protein. ELISA plates coated with the isolated 29-kDa protein were preincubated for 2 h at 37°C in the presence of various concentrations of human IgG Fc fragments (0

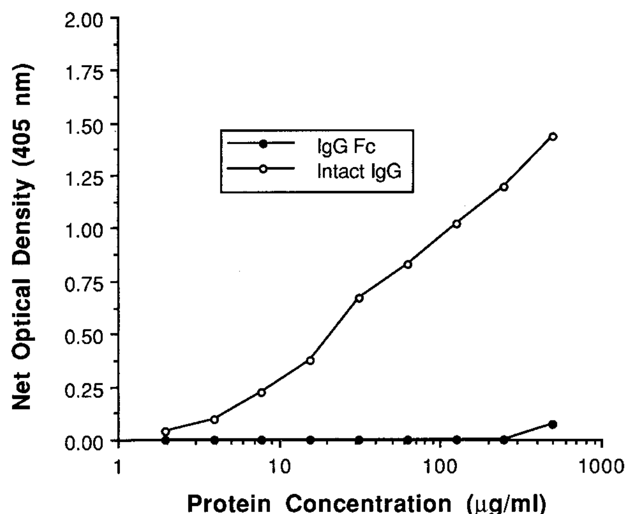

FIG. 4. Binding of intact polyclonal human IgG and IgG Fc fragments to the isolated 29-kDa protein of *A. actinomycetemcomitans*.

to 500 µg/ml), rinsed, and incubated with serial dilutions of high-titer LJP serum. Residual binding of IgG1 and IgG2 antibodies in LJP serum was quantified as described above. Preincubation of *A. actinomycetemcomitans* 29-kDa protein-coated plates with human IgG Fc fragments had no inhibitory effect upon subsequent binding of either IgG1 (Fig. 5) or IgG2 (data not shown) antibodies in LJP serum to this antigen. Fc fragments (at protein concentrations of >125 µg/ml) appeared to increase the binding of IgG1 and IgG2 antibodies, particularly at higher dilutions of serum. This was likely due to nonspecific binding of Fc fragments incompletely removed during the rinsing procedure.

IgG subclass response to the 16.6-kDa OMP. Previous studies to define the cell envelope antigens recognized by IgG antibodies in LJP sera indicated that in addition to the 29-kDa

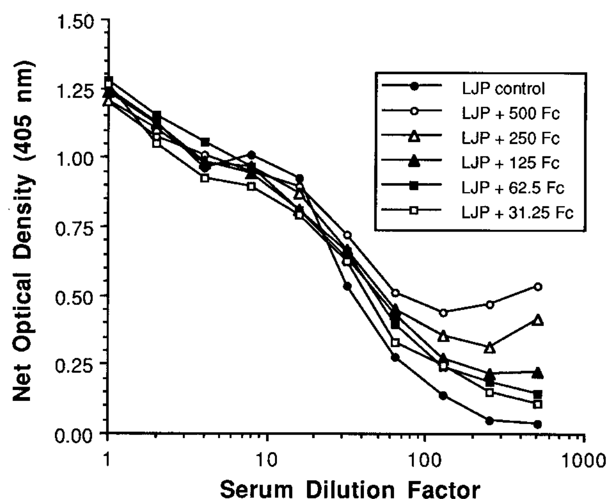

FIG. 5. Effects of preincubation of high-titer LJP serum with Fc fragments of human polyclonal IgG on the binding of IgG1 antibodies to the 29-kDa OMP. ELISA plates coated with the 29-kDa protein were preincubated for 2 h at 37°C in the presence or absence of human IgG Fc fragments. These plates were washed and incubated with serial dilutions of high-titer LJP serum. IgG binding was quantified as described in Materials and Methods with biotinylated murine anti-human IgG1.

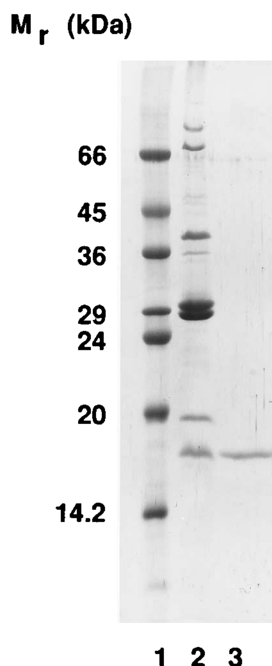

FIG. 6. Coomassie blue-stained SDS-polyacrylamide gel that depicts the isolated 16.6-kDa OMP of *A. actinomycetemcomitans* Y4. Lane 1, low-molecular-mass standards; lane 2, Sarkosyl-insoluble outer membrane fraction; lane 3, isolated 16.6-kDa protein. Samples were solubilized in sample buffer at 50°C prior to application to slots of a minigel that consisted of a 3% stacking gel and a 14% resolving gel.

protein, a protein that migrated with an apparent molecular mass of 16.6 kDa appeared to be a target for such antibody recognition (32). This protein, which was soluble in 1% SDS following incubation for 30 min at 60°C, was further purified by preparative SDS-PAGE. The isolated protein migrated with an apparent molecular mass of 16.6 kDa (Fig. 6, lane 3), similar to that of the corresponding protein in the Sarkosyl-insoluble outer membrane fraction (Fig. 6, lane 2). This protein was subsequently employed in the quantitative IgG subclass ELISA to determine the pattern of IgG subclass response to this OMP. Mean concentrations of IgG1 and IgG2 antibodies to the 16.6-kDa protein in sera were found to be significantly elevated for the LJP group, compared with those for a group of periodontally healthy, race-matched controls (Table 2). The IgG1 and IgG2 antibody responses of the LJP group to the 16.6-kDa protein did not segregate into high- and low-titer subgroups, as was the case with the 29-kDa protein; hence, mean concentrations of antibody to the 16.6-kDa protein were calculated for the LJP group as a whole.

In a comparison of the data in Tables 1 and 2, the 16.6-kDa protein evoked weaker IgG1, IgG2, and IgG3 antibody responses than those observed for the 29-kDa protein. Thirteen of 20 LJP sera tested had levels of IgG1 antibody to the 29-kDa protein which were at least twofold greater than antibody levels to the 16.6-kDa protein. Similarly, 17 of 20 LJP sera had levels of IgG2 antibody to the 29-kDa protein which were at least twofold greater than antibody levels to the 16.6-kDa protein. In contrast, IgG1 and IgG2 antibody levels to the 16.6-kDa protein exceeded (by at least twofold) those to the 29-kDa protein in only 2 of 20 sera tested for each subclass response. Hence, the 29-kDa protein appears to be quantitatively more important as a target antigen for IgG antibodies in sera from LJP patients than is the 16.6-kDa protein.

IgG subclass concentrations in sera from LJP patients and periodontally healthy individuals. The atypical IgG2 antibody response to the 29-kDa OMP seen in many of the high-titer LJP sera prompted us to determine if there were aberrations in the total IgG and/or IgG subclass concentrations in sera from these patients compared with those from low-titer LJP subjects and periodontally healthy, race-matched controls. As indicated in Table 3, the mean IgG2 concentration in serum was significantly elevated for the high-titer LJP group compared with those for the low-titer LJP and periodontally healthy groups. This increase was further reflected in a significant elevation in total IgG concentration in serum for the high-titer LJP group. In contrast, IgG1, IgG3, and IgG4 concentrations were within normal limits for this same group. There were no significant differences in the total IgG and IgG subclass concentrations in sera from low-titer LJP patients and periodontally healthy subjects.

DISCUSSION

There has been considerable interest in defining the major surface antigens of *A. actinomycetemcomitans* recognized by IgG antibodies in sera from periodontitis patients colonized by this organism. Much of the work in this area has focused upon characterization of the IgG antibody response to serotype-specific determinants in LPS, a major component of the outer membranes of gram-negative bacteria. It has been reported by several laboratories that the sera from LJP subjects contain markedly elevated levels of IgG antibody to LPS (27, 37), with the bulk of these antibodies belonging to the IgG2 subclass (18, 19, 36). However, the outer membrane of *A. actinomycetemcomitans* also contains a number of membrane proteins which are recognized by IgG antibodies in sera from periodontitis patients (3, 31, 32). To date, little attention has been directed to quantitative evaluation of the IgG subclass response to OMPs. Accordingly, the focus of this study was to define the distribution of IgG subclass antibodies directed to selected major OMPs of *A. actinomycetemcomitans*.

TABLE 2. Concentrations of IgG subclass antibodies to the 16.6-kDa OMP in sera from LJP patients and periodontally healthy race-matched controls

Patient group	IgG subclass antibody concn[a] (range)			
	IgG1	IgG2	IgG3	IgG4
LJP (n = 20)	3.33[b] (0.78–11.20)	1.11[c] (0.20–6.93)	0.33 (0.06–1.75)	<0.06
PH[d] (n = 6)	0.95 (0.27–7.27)	0.07 (0.04–0.24)	ND[e]	ND

[a] Geometric mean concentration in micrograms per milliliter.
[b] Significantly different from mean IgG1 concentration of the PH group ($P < 0.05$) by Wilcoxon-Mann-Whitney test.
[c] Significantly different from mean IgG2 concentration of the PH group ($P < 0.001$) by Wilcoxon-Mann-Whitney test.
[d] PH, periodontally healthy, race-matched control subjects.
[e] ND, not determined.

TABLE 3. Total IgG and IgG subclass concentrations in sera from LJP patients and periodontally healthy controls

Patient group	Concn (g/liter) in serum[a]				
	Total IgG	IgG1	IgG2	IgG3	IgG4
High-titer LJP (n = 10)	14.87[b] ± 3.02	8.17 ± 1.63	5.29[b] ± 1.84	0.87 ± 0.24	0.54 ± 0.29
Low-titer LJP (n = 10)	12.84 ± 2.80	8.73 ± 2.82	2.75 ± 1.06	0.99 ± 0.40	0.36 ± 0.23
Periodontally healthy (n = 8)	11.49 ± 2.68	7.06 ± 2.38	3.15 ± 1.15	0.68 ± 0.28	0.59 ± 0.52
Normal range[c]	3.8–15.0	1.8–7.8	1.0–4.6	0.3–1.4	0.08–1.8

[a] IgG subclass concentrations were determined by immunoenzymetric assay. Results are reported as arithmetic means ± standard deviations. Total IgG concentrations were determined by summation of individual IgG subclass concentrations. Total IgG concentrations estimated in this manner were within 15% of the IgG concentrations in serum as measured by radial immunodiffusion.
[b] Significantly different from low-titer LJP and periodontally healthy groups ($P < 0.01$).
[c] Normal values (95% confidence intervals) reported by Papadea and coworkers (24).

A number of membrane proteins of *A. actinomycetemcomitans* have been identified as targets for IgG antibodies in sera from periodontitis patients on the basis of immunoblot analysis (3, 31). One such protein is a 29-kDa species which exhibits heat modifiability and has been shown to exhibit N-terminal sequence homology with the OmpA family of proteins expressed in many species of gram-negative bacteria (33), including members of the genus *Haemophilus* (34). It has been estimated that the OmpA protein of *H. influenzae* constitutes roughly 25% of the total protein of the outer membrane of this species. Similarly, the 29-kDa protein appears to represent a major OMP of *A. actinomycetemcomitans* (3, 32). A second OMP, which migrates with an apparent molecular mass of 16.6 kDa, is also recognized by IgG antibodies in sera from periodontitis patients (3, 32). This protein exhibits solubility characteristics similar to those of the peptidoglycan-associated lipoprotein of *H. influenzae* (22) and is reactive with a monoclonal antibody (7F3) to a highly conserved epitope on the 16.6-kDa protein expressed by all typeable and nontypeable *H. influenzae* strains (23, 35). The peptidoglycan-associated lipoprotein has been estimated to constitute approximately 5% of the total protein of the outer membrane of *H. influenzae* (22).

It has previously been noted that black LJP subjects exhibit a higher prevalence of seropositivity for antigens of *A. actinomycetemcomitans* than do white LJP subjects (11). Moreover, black LJP subjects often exhibit the most exuberant IgG antibody responses to serospecific determinants in *A. actinomycetemcomitans* serotype b LPS (4, 37). Consistent with these findings, 13 of the 14 high-titer LJP sera used in this study were obtained from black subjects. On the other hand, the low-titer LJP group appears to be racially nonhomogeneous, including blacks, whites, and a single Asian. It has also been observed that periodontally healthy black subjects are characterized by a higher prevalence of seropositivity for *A. actinomycetemcomitans* antigens than are healthy white subjects (11). Hence, in evaluating the IgG subclass responses of high- and low-titer LJP subjects to isolated membrane proteins of *A. actinomycetemcomitans*, the data were compared with those obtained with sera from racially matched control subjects.

In this study, we have demonstrated that sera from LJP subjects contain significantly elevated levels of IgG1, IgG2, and IgG3 antibodies (but not IgG4 antibodies) to the 29-kDa OMP of *A. actinomycetemcomitans*. Many of these sera also contained significantly elevated levels of IgG1 and IgG2 antibodies to the 16.6-kDa protein, although these levels were substantially lower than those observed for the 29-kDa protein. The presence of elevated levels of IgG1 and, in the case of the 29-kDa protein, IgG3 antibodies to the isolated OMPs of *A. actinomycetemcomitans* was not surprising, as IgG antibody responses to protein antigens often involve these two subclasses. However, the IgG2 subclass response to the 29-kDa protein was unanticipated, as IgG2 antibodies are typically produced in response to carbohydrate antigens (28). Studies that employed affinity-purified IgG2 antibodies from sera of LJP subjects and periodontally healthy individuals confirmed the strong reactivity of LJP IgG2 with the isolated 29-kDa protein.

The high-titer LJP sera employed in this study had previously been found to contain substantial amounts of IgG2 antibodies reactive to the LPS of *A. actinomycetemcomitans* Y4 (36). This raised some concern as to whether the IgG2 antibodies detected by the OMP-specific ELISA were indeed specific for the 29-kDa protein or recognized LPS contaminants. However, several lines of evidence argue against the latter possibility. First, LPS was detected only in trace amounts in the isolated 29-kDa protein (as well as in the 16.6-kDa protein), as determined by *Limulus* lysate gelation and silver staining of SDS-polyacrylamide gels. Secondly, the LFO of *A. actinomycetemcomitans* Y4 LPS, a potent inhibitor of the IgG2 antibody response to LPS, had little effect on the IgG2 antibody response to the 29-kDa OMP (Fig. 3). Thus, the elevated IgG2 antibody response to the 29-kDa OMP cannot be explained on the basis of LPS contamination of the isolated protein.

We also considered the possibility that the IgG2 antibody response to the 29-kDa OMP might be attributable to the presence of a carbohydrate moiety in this molecule. However, the isolated protein was unreactive to staining with periodic acid-Schiff reagent and similarly unreactive in an assay for detection of glycoproteins. Acid hydrolysis did reveal the presence of small amounts of glucosamine and galactosamine. It is not clear, however, whether these amino sugars are N linked to the protein or are merely contaminants. In this context, it was initially reported that *E. coli* OmpA protein contained glucosamine (8). However, subsequent analysis of the primary structure of *E. coli* OmpA protein did not reveal the presence of amino sugars (5). Further study to determine if the OmpA protein of *A. actinomycetemcomitans* is glycosylated and if the presence of a carbohydrate moiety can explain the atypical IgG subclass response to this protein is needed.

It has been suggested that the 29-kDa protein of *A. actinomycetemcomitans* possesses IgG Fc-binding activity (21). However, we were unable to demonstrate the binding of human IgG Fc fragments to ELISA plates coated with the isolated 29-kDa protein. Moreover, IgG Fc fragments did not inhibit the binding of IgG1 or IgG2 antibodies in high-titer LJP serum to the 29-kDa protein. Thus, the isolated 29-kDa protein lacks Fc-binding activity, which might account for the significant amounts of IgG antibody reactive to this protein in our assay system.

We are unaware of other studies that have reported such a strong IgG2 subclass response to a protein antigen. However, much of our knowledge of the typical IgG subclass pattern to protein antigens is based on studies that employed toxins rather than integral membrane proteins (14, 15, 28). Moreover, previous studies that assessed the subclass distribution of IgG antibodies to protein antigens in general, and bacterial OMPs in particular, were of a qualitative or semiquantitative rather than a quantitative nature. The magnitude of the response may reflect the fact that the 29-kDa OmpA protein constitutes one of the principal OMPs of *A. actinomycetemcomitans* (as is the case for the homologous protein in *H. influenzae*) and is thus an immunologically significant surface antigen. OmpA proteins have been reported to exhibit strong mitogenic activity for murine B lymphocytes (2, 17). If the OmpA protein of *A. actinomycetemcomitans* exhibits similar mitogenic activity toward human B lymphocytes, such activity might serve to promote clonal expansion of B lymphocytes capable of producing antibodies to this antigen. It is unclear why the 29-kDa OmpA protein evoked such a strong IgG2 antibody response in many of the LJP subjects. This may be due to the nature of the OmpA protein itself. If so, a similar IgG subclass distribution might be observed with other members of the OmpA family. Alternatively, the LJP subjects that respond in this manner may be genetically predisposed to overproduction of IgG2 antibodies following exposure to both polysaccharide and protein antigens. Analysis of the IgG subclass response of these patients to other protein antigens, including tetanus and diphtheria toxoids, might shed light on this possibility.

It is intriguing that *A. actinomycetemcomitans* LPS elicits such a strong IgG (predominantly IgG2) antibody response in LJP patients colonized by this organism. Type 1 thymus-independent antigens such as LPS typically induce production of antibodies of the IgM isotype. However, it has been noted that the immunization of mice with complexes of LPS and bacterial OMPs can lead to a heightened IgG antibody response to LPS, accompanied by an alteration in the IgG subclass distribution to one in which IgG2 antibodies predominate (16). In that study, the authors noted that the 39-kDa OMP of *Proteus mirabilis*, which is now recognized as a member of the OmpA family of proteins (17), exhibited potent adjuvant activity in stimulating production of IgG2 antibodies to LPS (16). If the OmpA protein of *A. actinomycetemcomitans* exhibits similar adjuvant activity, this may explain the strong IgG2 antibody response to LPS (and to the OmpA protein itself) seen in LJP patients. This possibility warrants further investigation.

Many of the high-titer LJP sera evaluated to date have been found to contain substantial elevations in IgG2 antibodies to both LPS and the 29-kDa OMP of *A. actinomycetemcomitans*. This suggests that LJP subjects, especially blacks, may be hyperresponsive to certain antigens. Consistent with this hypothesis, we previously reported that high-titer sera predominantly from black LJP subjects contain significantly elevated IgG2 antibody concentrations (36). Employing sera from a larger number of high-titer patients in this study, we have confirmed this finding. Moreover, a recent study by Lu and coworkers (20) indicates that black subjects, regardless of periodontal status, have higher IgG2 antibody concentrations in their sera than do white subjects, with sera from black LJP subjects having the highest IgG2 levels. On the other hand, our results indicate that the levels of IgG1, IgG3, and IgG4 in sera were comparable for all groups (Table 3). The elevation in IgG2 concentration was also reflected in a significant rise in the total IgG concentration for the high-titer group.

LJP sera have been demonstrated to contain opsonic IgG antibodies to *A. actinomycetemcomitans* (1). The importance of OMPs as putative targets for such opsonic activity has not been defined. It has been observed, however, that antibodies to the major OMPs of *Actinobacillus pleuropneumoniae*, which include a 29-kDa species, appear to be opsonic for this organism (29). We are currently examining the role of IgG antibodies directed to the 29-kDa protein in opsonization of *A. actinomycetemcomitans*.

The results of this study, together with earlier results (36), indicate that sera from LJP patients colonized by *A. actinomycetemcomitans* contain substantial amounts of IgG2 antibodies directed to cell envelope determinants of this organism. These antibodies appear to be directed not only to LPS-associated determinants but also to at least one principal OMP. Therefore, we have performed studies to ascertain whether IgG2 antibodies against somatic antigens of *A. actinomycetemcomitans* express functional properties which may be beneficial in the eradication of this organism, particularly in regard to opsonophagocytosis. Elsewhere we provide direct evidence that affinity-purified IgG2 antibodies prepared from high-titer LJP sera are opsonic for *A. actinomycetemcomitans* (35a). Hence, LJP subjects colonized by *A. actinomycetemcomitans* mount a humoral response which includes the production of substantial amounts of IgG2 antibodies capable of augmenting phagocytic uptake of this periodontopathic organism. Studies to define the antigen(s) recognized by such opsonic IgG2 antibodies are in progress.

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