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Anticomplementary Activity of *Fusobacterium polymorphum* in Normal and C4-Deficient Sources of Guinea Pig Complement

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Fusobacterium polymorphum has been isolated from the gingival crevice in humans and has been implicated in the immunopathology of periodontal diseases. The presence of alternative complement pathway factors in gingival crevice material suggests the contribution of this process in the manifestation of the local host response. A complement consumption assay was used to show that the anticomplementary activity of a cell-wall preparation from *F. polymorphum* in guinea pig complement progressed optimally at 37°C and suboptimally at 0°C. Similar levels of complement consumption were recorded at both temperatures, but a higher concentration of cell walls was required to demonstrate maximum activity in the cold. Augmentation experiments with additional normal and heat-treated complement showed the presence of heat-labile factors that controlled and limited the consumption of complement to a constant proportion of the total amount present. These same experiments indicated that there were no reactive antibodies in the complement used in the assay. Similar tests with C4-deficient guinea pig sera confirmed that *F. polymorphum* cell walls were capable of generating alternative complement pathway activity in guinea pig sera.

Gram-negative filamentous bacteria belonging to the genus *Fusobacterium* tend to increase numerically with time in dental plaque (21-23). In addition, recent reports indicate that *F. polymorphum*, also designated *F. nucleatum* (16), has been determined as one of the major anaerobic isolates from advanced cases of periodontitis (35, 41; A. A. Crawford, S. S. Socransky, and G. Bratthall, J. Dent. Res. **54A**:97, 1975). The discovery that serum antibodies are reactive with oral gram-negative anaerobic organisms has implicated these indigenous crevicular microorganisms in the immunopathology of periodontal diseases (6, 12, 15, 18, 19, 26). This paper is a continuation of our investigations into the involvement of *F. polymorphum* as a potential etiological agent in human periodontal disease.

It has been suggested that the histopathology of periodontal diseases is mediated through the participation of immune recognition and effector systems (17). One effector is the complement system, which is classically activated by the combination of immune complexes and the early complement components (9, 28). The products of the classical pathway have shown chemotactic, anaphylatoxic, and opsonic activity in vitro (4). The complement system may also be activated by mechanisms not involving specific immunoglobulins or the early complement components. This alternative pathway is initiated by the reaction of certain polymeric substances (inulin, endotoxin, or zymosan) with specific non-immunoglobulin serum factors (13, 14, 29, 30, 31, 33, 34, 36, 40). The subsequent cleavage of C3 and the generation of biologically active complement fragments may also be important to the host response mechanisms in inflammatory disease states.

Bladen et al. have demonstrated the appearance of lytic lesions on the surface of *Veillonella alkalescens* and lipopolysaccharides (LPS) when exposed to guinea pig (GP) sera. It was further determined that complement was consumed primarily between C3 and C9 and that the reactions were characteristically inefficient at 0°C but optimally efficient at 37°C (3). Gewurz et al. established that LPS, zymosan, and flagellin produced reduction of total complement activity in GP sera (11). They later reported that the anticomplementary activity of LPS was directed toward the terminal components, C3-C9, and suggested that the activation of C1, C2, and C4 may have occurred, but changes in hemolytic levels of these components could not be detected outside the sensitivity limits of the hemolytic assay (10).

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Marcus et al. (24) attempted to resolve the issue of early component participation in the anticomplementary activity of LPS by preincubating LPS with GP sera. The resulting complex of LPS and serum factors, "LPS-X," consumed C3, and anti-C2 did not interfere with this alternative pathway activity. Phillips et al. (32) indicated that LPS, when bound to the surface of erythrocytes as E-LPS, would only activate the complement cascade via the classical pathway. Later, it was revealed by Mergenhagen et al. (27) that different preparations of LPS had demonstrated the capability of initiating complement consumption by either the classical or the alternative complement pathways.

With the discovery of a strain of guinea pigs with a genetic deficiency in C4 (C4D), it was believed that sera obtained from these animals could be used to resolve the proposed alternative pathway activity by LPS (5). Subsequently, sera from C4D guinea pigs have been effectively used to examine the activation of the alternate pathway by LPS and pneumococcal polysaccharides (7, 42).

Previous studies suggest that the ingredients (LPS and complement factors) for alternative pathway activation of the complement system are present in the diseased gingival crevice (2, 37, 39). There is also evidence that alternative pathway activation has occurred in inflamed periodontal tissues (1). We present evidence here that the cell walls obtained from the gram-negative anaerobe *F. polymorphum*, a major isolate of the diseased gingival crevice, are capable of activating the alternative complement pathway.

MATERIALS AND METHODS

Growth of microorganisms. *F. polymorphum* organisms (ATCC 10953) were grown under anaerobic conditions for 48 h at 37°C by an anaerobe jar-Gaspak system (Baltimore Biological Laboratory [BBL], Cockeysville, Md.). A liquid modified tryptone medium (pH 7.2) was used containing (per liter of distilled water): tryptone (Difco), 10 g; yeast extract (Difco), 10 g; K_2HPO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 1.25 g; glucose, 2 g; and sodium thioglycolate (BBL), 5 g.

Preparation of cell walls. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed three times with 0.01 M phosphate-buffered saline (PBS) (0.15 M NaCl; pH 7.2). The cells were then suspended in isotonic saline and sonically treated in a dry ice-alcohol bath using a Branson sonic oscillator with a probe tip at 6 A and 8 bursts of 30 s each. The cell-wall preparation of *F. polymorphum* was derived from the sonically treated cellular debris according to the modified methods of Garcia et al. (8). Briefly, the sediment obtained at 2,000 × *g* for 10 min was suspended in distilled water and then washed five times in distilled water at 20,000 × *g* for 15 min until the wash supernatants showed no absorbance at 260 nm. Before they were used in the complement consumption assay, lyophilized cell walls were reconstituted to a concentration of 5,000 μg (dry weight) per ml in deionized and distilled water and designated FP cell walls.

Complement titration. Complement titration was performed according to a modified version of the LBCF method (20). Modification included using 5 g of dextrose per liter of Veronal-buffered diluent (VBD). VBD prepared with the 5× stock buffer containing Mg^{2+} and Ca^{2+} was designated VBD^{2+}. A similar 5× stock buffer solution was also prepared containing 42.5 g of NaCl with no stock divalent cation solution and was used in the preparation of VBD^{2-}.

Complement consumption assay in normal GPC. The methods of Bladen et al. (3), Gewurz et al. (10), and Philips et al. (32) were employed as the basis for the complement consumption assay. The test consisted of placing 0.1 ml of GP complement (GPC), 0.1 ml of either the experimental cell-wall preparation (500 μg) or VBD^{2+} as the negative control, and 0.8 ml of VBD^{2+} into a serological test tube (12 by 75 mm). Inulin was used in the assay at 500 μg/ml as a positive control. The system was incubated in a water bath for 1 h at 37°C or incubated in an ice bath for 1 h at 0°C. Variations in the basic assay with GPC included testing the effect of from 100 to 1,000 μg of the cell walls on a fixed amount of GPC and testing the effect of twofold and fourfold increases in normal and heat-treated (56°C for 30 min) GPC with a standard amount (125 μg) of cell walls.

At the end of the incubation period, the serological tubes were placed in an ice bath, and a complement titration was performed on dilutions of the VBD^{2+} controls (20). The number of 50% hemolysis complement (CH_{50}) units in the original 1-ml sample of undiluted GPC was determined. From this value, the number of CH_{50} units contained in 0.3 ml of the assay mixtures could be calculated. Subsequently, 0.3 ml of the diluted and undiluted tests was added to 0.5 ml of VBD^{2+}. Then, 0.2 ml of the 3% suspension of hemolysin-sensitized sheep erythrocytes (EA) was added, and the mixture was incubated in a water bath at 37°C for 30 min. The degree of hemolysis seen in the control and experimental tests was evaluated by single-point analysis of partial lysis to determine residual complement activity. Conversion factors calculated from the Von Krogh equation were used in these computations (25). All consumed or missing CH_{50} unit activity was expressed as the absolute difference or the percent difference between the hemolytic complement activity determined in controls and experimentals by single-point analysis and the same activity determined in VBD^{2+} controls by complement titration.

Complement consumption assay of C4-deficient guinea pig sera. The complement consumption assay described above was modified to test the reduction of total hemolytic complement activity in C4-deficient GP sera (C4D). C4D was kindly provided by Jerry A. Winkelstein (Department of Pediatrics, The Johns Hopkins School of Medicine, Baltimore, Md.) and was taken from descendants of those animals determined to have a genetically based deficiency in C4 (5, 7, 42).

EAC1,4 were prepared by the methods of Mayer (25). Briefly, sheep erythrocytes were washed three

times in VBD^{2-} supplemented by 0.001 M CaCl. A 3.0% suspension of these cells was sensitized with hemolysin (EA) (20). GPC (0.35 ml) was added to 7.0 ml of EA and allowed to react for 30 min at 0°C. The cells were then washed twice with the VBD^{2-}–0.001 M $CaCl_2$ buffer, suspended in 14 ml of the same buffer, incubated for 90 min at 37°C, and sedimented by centrifugation in the cold. The resulting EAC1,4 were suspended in VBD^{2-} and stored at 0°C until their use in the complement consumption assay with C4D. Before they were used in the test, the EAC1,4 were washed once in VBD^{2+}, and 50 μl of a 3.0% suspension was tested with an equal volume of either C4D, GPC, or VBD^{2+} to determine the potential ability of these cells to lyse under test conditions.

In the complement consumption assay system, 0.1 ml of C4D was placed in a serological tube (12 by 75 mm) with 0.1 ml of either the cell-wall preparation, inulin, or VBD^{2+}. After the addition of an additional 0.8 ml of VBD^{2+}, the test mixture was incubated in a water bath for 1 h at 37°C. At the end of the incubation period, the reaction mixtures were placed in an ice bath, and a complement titration was performed on the VBD^{2+} negative controls using the EAC1,4 cells. The experimentals, the inulin controls, and the VBD^{2+} controls were then evaluated for residual complement activity using the EAC1,4. Single-point analysis of partial lysis was used to establish the degree of hemolysis, and the Von Krogh conversion factors were employed to compute the CH_{50} unit activity (25). In addition, the same test solutions were examined with EA as a control for classical pathway in C4D. The results of the complement consumption assay with C4D were expressed as a percent reduction in the total CH_{50} units that had been established in the VBD^{2+} controls by complement titration.

RESULTS

Complement consumption assay of normal GPC. *F. polymorphum* cell walls at dry weights of 31.25, 62.5, 125, 250, 500, and 1,000 μg were tested in the complement consumption assay with 28.14 CH_{50} units of GPC at 37°C for 1 h. Figure 1 indicates that 31.25 and 62.5 μg of the cell walls showed minimal consumption of complement activity. Increasing the concentration to 125 μg produced a sharp elevation in the number and percentage of CH_{50} units consumed. The increase between 250 and 500 μg was more gradual, reaching an apparent optimum consumption of 50.6%. There was no increase in anticomplementary activity of the cell walls when 1,000 μg was used in the test.

A similar titration was performed at an incubation temperature of 0°C for 1 h. There was a gradual increase in complement consumption with increasing concentrations of cell walls in the assay (Fig. 2). The lower temperatures caused consumption of 12.2 and 20.2% with 500 μg and 1,000 μg, respectively. Increasing the concentration of cell walls to 2,000 μg produced complement consumption to a level of 60.1%.

The complement consumption assay was used

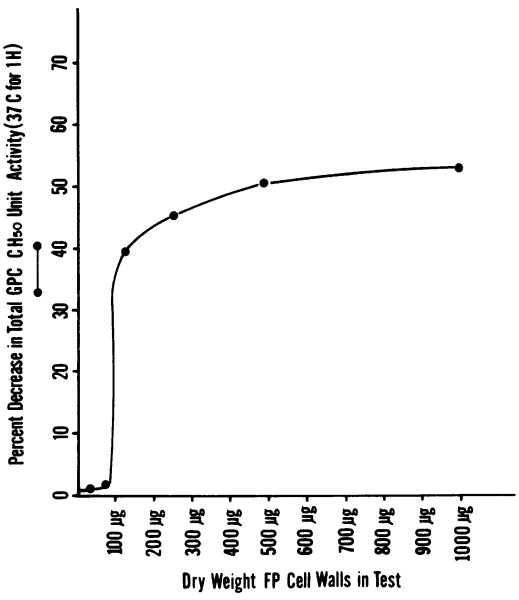

FIG. 1. *Anticomplementary effect of different concentrations of cell walls on a fixed amount of GPC (28.14 CH_{50} units). The curve represents the percent units consumed (●) by 31.25, 62.5, 125, 250, 500, and 1,000 μg (dry weight) of the FP cell-wall preparation.*

to determine the heat stability of factors in GPC, which might augment the reaction of a suboptimal concentration of the cell walls. For this purpose, a 125-μg amount was chosen since it had been shown in earlier tests to reduce the hemolytic complement levels by only 39.7% compared with a maximum of greater than 50% with 500 μg. The basic test contained 33.33 titratable CH_{50} units of GPC. The test was augmented with either 33.33 or 99.99 additional CH_{50} units of normal or heat-treated (56°C for 30 min) GPC. Table 1 indicates that increasing CH_{50} units of GPC were consumed by the standard 125 μg of cell walls as more normal GPC was put into the test. With a twofold increase in units present, there was an approximate twofold increase (14.64 to 27.06) in units consumed. Similarly, with a fourfold increase in CH_{50} units present, there was a slightly greater than fourfold increase (14.64 to 68.28) in consumption. In the tests where the heat-treated GPC was added, there was no detectable increase in CH_{50} unit consumption.

Complement consumption assay of C4D. EAC1,4 cells at a 3% suspension in VBD^{2+} were tested for their ability to lyse in the presence of either GPC or C4D. A 3% suspension of EA was tested in the same manner. All EA cells lysed in 20 min at room temperature in the presence of normal GPC; however, EA did not lyse in C4D or VBD^{2+} and formed a button in 1 h. In contrast, all EAC1,4 cells lysed in both normal

GPC and C4D in 20 min at room temperature. The results of paired complement consumption in normal GPC and C4D are shown in Table 2. *F. polymorphum* cell walls at a concentration of 500 μg in the test were capable of reducing the complement activity by 44.5% in normal GPC and by 32.4% in C4D. Inulin also consumed complement activity from both sera, 48.7% from normal GPC and 22.3% from C4D. The ability of EA cells to lyse in normal GPC was inhibited in C4D by more than 90%.

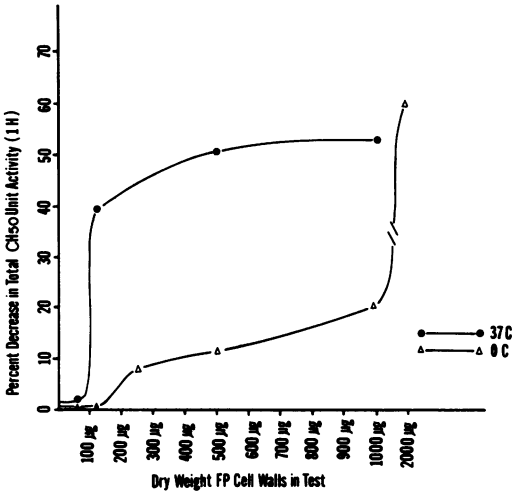

FIG. 2. *Effect of varying concentrations of cell walls using 62.5 to 2,000 μg (dry weight) in the complement consumption assay with normal GPC. The lower curve (Δ) represents the percent consumption of 40.26 CH_{50} units at 0°C.*

TABLE 1. *Consumption of GPC by 125 μg of F. polymorphum cell walls supplemented with normal or heat-treated GPC*

No. of CH_{50} units in test	Units consumed/ total units present in test	Consumption (%)	Additional units consumed
33.33	14.64/33.33	43.9	
66.66	27.06/66.66	40.6	12.42
133.32	68.28/133.32	51.2	53.64
66.66[a]	14.64/33.33	43.9	0
133.32[b]	14.64/33.33	43.9	0
33.33[c]	18.33/33.33	55.0	
33.33[d]	20.00/33.33	60.2	
VBD[e]	0.63/33.33	1.9	

[a] 33.33 of the 66.66 units present had been heat treated before use in the complement consumption assay.

[b] 99.99 of the 133.32 units present had been heat treated before use in the complement consumption assay.

[c] Control with 500 μg of cell walls.

[d] Control with 500 μg of inulin.

[e] Control showing difference between the number of CH_{50} units in VBD controls by complement titration and by single-point analysis of partial lysis.

TABLE 2. *Consumption of CH_{50} units in C4-deficient GP sera*

Anticomplementary agent/ complement source	Units consumed/ total units present in test	Consumption (%)
Cell wall/GPC (500 μg)	8.34/18.15	44.5
Cell wall/C4D (500 μg)	10.17/31.35	32.4
Inulin/GPC (500 μg)	9.14/18.75	48.7
Inulin/C4D (500 μg)	6.99/31.35	22.3
EA/GPC/VBD[a]	1.25/18.15	6.7
EAC1,4/C4D/VBD[b]	2.29/31.35	7.3

[a] Control showing difference between the number of CH_{50} units in GPC-negative VBD controls as determined by complement titration and by single-point analysis of partial lysis.

[b] Control showing difference between the number of CH_{50} units in C4D-negative VBD controls as determined by complement titration and by single-point analysis of partial lysis.

A test using C4D sera was performed to determine if increased consumption could be recorded by increasing the amount of cell walls in the test from 125 to 1,000 μg. The results of the tests in which sera were incubated for 1 h at 37°C are shown in Table 3. Complement was consumed with increasing concentrations of cell walls in the test.

DISCUSSION

Based on earlier tests that indicated that the anticomplementary activity of *F. polymorphum* might be localized in cell-wall fragments (C. E. Hawley and W. A. Falkler, Jr., manuscript in preparation), it was decided to select the cytoplasm-free cell-wall preparations as a standard anticomplementary agent. It also seemed logical that a study of the anticomplementary activity by the cell walls of *F. polymorphum* would have more relevance to the pathogenesis of clinical disease than standard laboratory preparations of LPS.

Bladen et al. (3) added LPS to a fixed amount of GPC and showed that between 100 and 200 μg of the LPS preparation would achieve maximum consumption of the CH_{50} unit activity at 37°C. A similar interaction between zymosan and human C3 was reported by Pillemer et al. (34). This previously described sigmoidal relationship was also demonstrated here by titrating *F. polymorphum* cell walls with a fixed amount of GPC. The results of the titration (Fig. 1) suggested that maximum anticomplementary activity occurred at 500 μg.

FP cell walls were also titrated with GPC for 1 h at 0°C. In contrast to the earlier reports by Bladen et al. (3) and Pillemer et al. (33, 34) there was a low level of CH_{50} unit consumption in these tests that ranged from 0% for 62.5 μg to 20.1% for 1,000 μg of the FP cell walls. The discrepancy between the results reported here

TABLE 3. *Consumption of CH_{50} units in C4-deficient GP sera*

Anticomplementary agent	Units consumed/ total units present in test	Consumption (%)
FP cell wall (125 μg)	19.34/51.93	37.2
FP cell wall (250 μg)	25.76/51.93	48.6
FP cell wall (1,000 μg)	37.36/51.93	73.1
EAC1,4/C4D/VBD[a]	0.99/51.93	1.9

[a] Control showing the difference between the number of CH_{50} units in C4D-negative VBD controls as determined by complement titration and by single-point analysis of partial lysis.

and the earlier reports of no measurable consumption to 0°C is unresolved by this investigation. However, our data suggest that the observed complement consumption was apparently influenced by the degree of interaction between the cell walls and GPC, which was concentration and temperature dependent.

It was determined during augmentation testing that the consumption of GPC by the cell walls of *F. polymorphum* was limited either by one or more of the heat-labile complement components (C1, C2, C3, C4) or perhaps by the heat-labile non-immunoglobulin serum factors (C3 proactivator) essential for the alternative pathway (13, 14, 25). This was shown by testing the consumption of normal GPC by a suboptimal concentration of cell walls in the presence of two- and fourfold additions of heat-treated or normal GPC. The use of suboptimal concentrations of FP cell walls (125 μg) was considered critical so that sufficient complement would be available to detect complement activation by heat-stable complement-fixing antibodies in the added heat-treated GPC. The selection of cell wall concentrations of less than 125 μg/ml was not used in the augmentation experiments because of the difficulties experienced in obtaining consistent base line consumption data in the 100 μg range (Fig. 1). The presence of unconsumed complement activity in 125 μg of cell walls was suggested by the higher levels of consumption shown with 500 μg of cell walls. The fact that the two- and fourfold additions of heat-treated GPC did not augment its consumption suggested that GPC did not contain antibody that could have reacted with cell walls and ultimately generated classical pathway consumption.

Whereas the alternative pathway of complement consumption by laboratory preparations of *F. polymorphum* were suggested in the tests with normal GPC, the confirmation of strict alternative pathway activity would require testing with C4D (5, 7, 42). Table 2 reveals significant anticomplementary activity by both cell walls and inulin at 500 μg each in the complement consumption assay with C4D. It was further shown here that EA cells could not lyse in the presence of the same dilutions of C4D that did lyse EAC1,4. This indicated that the classical complement pathway could not have functioned during the incubation of anticomplementary agents with C4D. An alternative complement pathway was confirmed through the use of C4D. As with GPC, progressive increases in C4D consumption were also recorded with increasing concentrations of cell walls in the assay.

On the basis of the findings reported here, the potential for activation of the alternative complement pathway by factors in the cell walls of *F. polymorphum* was supported. The exclusive activation of the alternative pathway without the influence of early complement components was confirmed with C4D. The potential for similar anticomplementary activity in situ and the generation of phlogistic active complement components by *Fusobacterium* species as part of the immunopathology of periodontal diseases may occur, but further investigations demonstrating the production of such factors will be required.

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