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THE ANTICOMPLEMENTARY ACTIVITY OF LIPO-POLYSACCHARIDE PREPARATIONS AND SONICATES FROM FUSOBACTERIUM POLYMORPHUM

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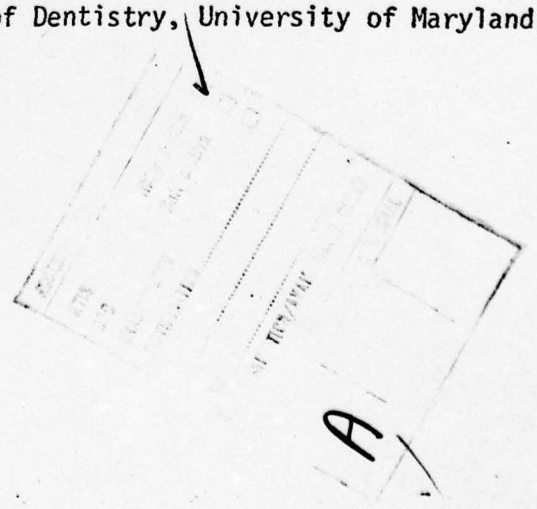

ABSTRACT

A complement consumption assay was employed to test the consumption of guinea pig complement (GPC') by different sonicate and lipopolysaccharide (LPS) preparations obtained from Fusobacterium polymorphum. The consumption of normal GPC' was observed at 37 C and varied depending upon the preparation used. All LPS preparations showed minimal activity at the concentrations employed in the assay. The observed anticomplementary activity was less potent at 4 C than at 37 C. Similar anticomplementary patterns were observed when inulin, a recognized activator of the alternate complement pathway, was tested in the complement consumption assay. The reactions of a sonicate preparation of F. polymorphum with GPC' and hyperimmune rabbit antisera were not affected by the lower temperatures. The addition of heat treated (56 C and 30 min) GPC' did not enhance the complement consumption displayed by a sonicate of F. polymorphum. It is therefore suggested that this organism is capable of alternate pathway activity in GPC'. Further tests revealed that the factors associated with the observed anticomplementary activity were resistant to the effects of heat (100 C for 30 min) and were located in the cell wall fractions of the sonicates. The cell walls also displayed endotoxic activity in a Limulus lysate assay. Because of these findings and the consistency of data produced by the cell walls at 37 C, it is concluded that the use of a cell wall preparation might be preferable to LPS in future investigations of this organism with the complement system.

INTRODUCTION:

Gram negative anaerobic filamentous bacteria are major components of subgingival bacterial plaque (Listgarten, Mayo, and Tremblay 1975). Specific environmental factors of the gingival crevice have been implicated in the creation of a unique eco-system which is essential for the growth of these generally fastidious organisms (Loesche 1969). In cases of advanced periodontitis, the non-spore forming, butyrate positive, gram negative anaerobic bacterium, Fusobacterium polymorphum (nucleatum) has been one of the major isolates from the gingival crevice (Sabiston and Grigsby 1972; van Palenstein Helderman 1975; Williams, Pantalone, and Sherris 1976). Lipopolysaccharide (LPS), extracted from the gram negative cell walls of Veillonella alcalescens (Bladen, Gewurz, and Mergenhagen 1967); Salmonella minnesota (Snyderman, Gewurz, and Mergenhagen 1968); Salmonella species, Arizona species, and Escherichia coli (Galanos et al. 1971) has demonstrated anticomplementary activity in vitro, and it has been suggested that this anticomplementary activity may occur via a preferential attack on C'3-C'9 (Gewurz et al. 1968; Marcus, Shin and Mayer 1971). However, the involvement of early complement components and reactive antibodies in the anticomplementary activity of LPS has not been ruled out (Phillips, Snyderman, and Mergenhagen 1972) and may depend upon the manner in which the LPS is presented to the complement system (Fine et al. 1972) and the availability of complement fixing antibodies (Snyderman and Pike 1975).

It has already been proposed that LPS may play an important role in the pathogenesis of periodontal disease (Berglund, Rizzo, and Mergenhagen 1969). Recent studies on the LPS content of crevicular fluid indicate that its endotoxic activity correlated well with the degree of clinical and

histologic inflammation in periodontal tissues (Simon et al. 1970; Simon et al. 1971). Simon et al. (1972) have further shown that the number of anaerobic gram negative rods in gingival exudates correlated with the amount of endotoxin in crevicular fluid and the degree of tissue inflammation. Schwartz, Stinson, and Parker (1972) reported that isotopically labelled endotoxin was capable of penetrating intact gingival crevicular epithelium. Sharpiro et al. (1972) also found the concentration of endotoxin in crevicular fluid elevated in association with increased histologic periodontal inflammation. A similar correlation was observed between the amount of endotoxin in dental plaque and the degree of histologic inflammation.

Immunoglobulins and complement components, including C'3, have been detected in inflamed gingival tissues by immunofluorescent techniques (Genco et al. 1974), and Shillitoe and Lehner (1972) have also found C'3 in crevicular fluid. Both suggest the involvement of immune complexes in the host response to crevicular bacteria. Attstrom et al. (1975) recently reported the presence of C'3, C'4 and C'5 in inflamed periodontal tissue. C'3 activator (Factor B), was also detected in gingival crevice material associated with diseased tissue. This evidence supports the concept of alternate complement pathway activity in the gingival crevice which may be mediated by bacterial polymers, such as LPS, acting on C'3 via the non-immunoglobulin serum factors of the Properdin system (Osler and Sandberg 1973). The generation of phlogistic factors by this process may be partially responsible for the vascular and cellular events seen in periodontal inflammation. We present evidence here that F. polymorphum, an organism prevalent in advanced periodontitis, may display alternate complement pathway activity.

MATERIALS AND METHODS:

Preparation of Microorganisms: Fusobacterium polymorphum (ATCC # 10953) was grown anaerobically, harvested, and sonicated according to the methods of Falkler and Hawley (1975). Briefly, the cells were incubated in a modified tryptone medium at 37 C for 48 hr. using the Gas-Pak system (Baltimore Biological Laboratories, Cockeysville, Md.). After incubation, the cells were harvested by centrifugation at 10,000 x g for 10 min at 4 C and washed three times in 0.01 M phosphate buffered saline, 0.15 M NaCl, pH 7.2 (PBS). The washed cells were resuspended in saline and disrupted ultrasonically at 6 AMPS in a dry ice/ethanol bath using 8 30 sec bursts.

Sonicate preparations of the organism were obtained according to the following protocol. The sonicate was first centrifuged at 2,000 x g for 10 min at 4 C. The supernatant was centrifuged again at 10,000 x g for 10 min at 4 C. The sediment at 10,000 x g was resuspended in isotonic saline and designated FP 2000. The supernatant was designated FP 10000. Aliquots of FP 2000 were used for the preparation of FP 2000 cell walls according to the methods of Garcia, Charlton, and Kay (1975) which included distilled water washes at 20,000 x g for 15 min until the wash supernatant showed no absorbance at 260 nm. The sediment from the initial 2,000 x g separation of the sonicate was also washed in the same manner and designated FP cell walls. FP 2000 and FP 10000 were tested for total protein by the Lowry-Ciocalteau method (Lowry et al. 1951) and frozen at -20 C. FP cell walls and FP 2000 cell walls were lyophilized, and the dry weight of each was determined.

FP cell walls were used in the phenol/water extraction of lipopolysaccharide as described by Weir (1969). A 6% cell wall suspension in distilled water was stirred in a water bath at 65 C. An equal volume of preheated 90% phenol was added and stirred for 5 min at 65 C. The mixture was cooled and centrifuged in the cold at 2,000 x g for 1 hr. The upper aqueous

phase and middle phenol phase were removed and dialyzed overnight in running tap water. Each phase was concentrated in carbowax (polyethylene glycol 6000), dialyzed at 4 C for 48 hr., lyophilized, and weighed for dry weight determination. The percent lipopolysaccharide yield from the cell wall preparations was 2.8% for the water phase LPS (WLPS) and 1.4% for the phenol phase LPS (PLPS). WLPS and PLPS were obtained in the same manner from acetone dried whole F. polymorphum cells (Weir 1969). The lipopolysaccharide yields were 6.1% for WLPS and 1.3% for PLPS. Aliquots of WLPS were purified by centrifugation at 100,000 x g for 2 hr. (Weir 1969). The resulting gelatinous pellet was resuspended in distilled water, lyophilized, and weighed. This preparation was designated FP LPS.

Preparation of Antisera: New Zealand white rabbits weighing 3-4 kg. were injected subcutaneously on the back with 325 µg of FP 10000 suspended in Fruend's Complete Adjuvant. This injection was repeated seven days later at a different subcutaneous site. These injections were followed by subcutaneous injections of 650 µg of FP 10000 without adjuvant at three weekly intervals. After a period of eight days, the animals were ear bled. The serum was separated from the clots by centrifugation and designated anti-FP serum. The serum was stored at -20 C.

Hemolysis Test: A microtiter hemolysis test using untanned sheep red blood cells (SRBC) was performed according to previously described methods (Hawley and Falkler 1975). FP 10000 at 1,700 µg/ml was incubated with an equal volume of a 2.5% PBS washed SRBC suspension at 37 C for 30 min. After washing the cells with cold PBS, the sensitized cells (50 µl) were placed in a microtiter plate with 50 µl serial dilutions of anti-FP serum which had been previously heat treated at 56 C for 30 min and absorbed at 37 C for 30 min with 50% SRBC suspensions. To each of the wells was added 25 µl of a 1:10 dilution of guinea pig complement (GPC') in PBS. The

plates were sealed and mixed for 1 min, incubated for 90 min at 37 C, and placed in the cold at 4 C for 18 hr. before reading. Controls for the hemolysis test included the reaction of unsensitized SRBC, antisera, and GPC' as well as the reaction of sensitized SRBC with GPC' in the absence of antisera.

Complement Consumption Assay: The methods of Bladen et al. (1967); Gerwurz et al. (1968); Gewurz et al. (1968); and Phillips et al. (1972) were used as the basis for the complement consumption assay. GPC' (Baltimore Biological Laboratories, Cockeysville Md.) was reconstituted, and 0.1 ml was placed in 12 x 75 mm serologic tubes. To this was added 0.1 ml of the experimental bacterial preparations or 0.1 ml veronal buffered diluent (VBD^{++}) as a negative control. The final test volume of 1.0 ml was made up with 0.8 ml $VBD.^{++}$ Positive controls of the assay included the use of 10 anticomplementary units of cobra venom factor (CoF) (Cordis Laboratories, Miami, Fla.) or 500 µg inulin (Fisher Scientific Co., Fairlawn, N.J.). The system was incubated in a 37 C water bath for 1 hr., or 1 to 18 hr. at 4 C in an ice bath. In some instances, parallel tests were performed to determine the effect of adding 0.1 ml heat treated (56 C for 30 min) GPC' or 0.1 ml heat treated and SRBC absorbed anti-FP serum to the complement consumption assay. At the end of the incubation period, the tubes were placed in an ice bath, and a complement titration was performed on the VBD^{++} negative controls following the protocol proposed by the Laboratory Branch Task Force (1965) using a 3.0% suspension of hemolysin sensitized SRBC (EA) in VBD^{++}. The correct dilution and the volume of that dilution which contained 1 $C'H_{50}$ unit were used to calculate the number of $C'H_{50}$ units in 0.3 ml of the test system. This value was established as the control level of hemolytic complement activity. The residual GPC' in the experimental and control tests was measured by single point analysis of

partial lysis using the conversions calculated from the Von Krogh equation (Mayer 1971). A 0.1 ml sample from each experimental and positive control was diluted to the same degree as the VBD^{++} control in the complement titration. Two other more concentrated dilutions were made to detect reduced $C'H_{50}$ unit activity. Each dilution (0.3 ml) was added to a serologic tube containing 0.5 ml VBD^{++}. To this mixture was added 0.2 ml sensitized SRBC. Each tube was shaken and incubated in a water bath at 37 C for 30 min. The tubes were centrifuged at 600 x g for 6 min and the degree of hemolysis was compared with hemoglobin standards. The degree of lysis was converted to a Von Krogh factor and multiplied by the dilution of the test showing lysis. This calculation indicated the number of $C'H_{50}$ units in 0.3 ml of the tests. Differences between the number of $C'H_{50}$ units in 0.3 ml of the VBD^{++} negative controls established by titration and the amount determined in the same volume of positive controls and experimentals by single point analysis of partial lysis were expressed as the number of units consumed or rendered non-titratable by agents in the test.

Limulus Amebocyte Lysate Test: The Limulus amebocyte lysate test was performed to detect endotoxin activity in F. polymorphum acetone dried whole cells, FP cell walls, LB cell walls (from Leptotrichia buccalis, prepared according to methods of Garcia, Costerton, and Kay 1975), WLPS, and PLPS. The E-Toxate system (Sigma Chemical Co., St. Louis, Mo.) was used for this purpose. All glassware used in the assay were rendered pyrogen free by autoclaving the materials at 121 C for 1 hr. and heating in a dry heat oven at 175 C for 8 hr. This assay for endotoxic activity will detect 1 ng Klebsiella endotoxin reference in 1 ml pyrogen free water.

RESULTS:

The results of the hemolysis test showed that the anti-FP rabbit sera

reacted with FP 10000 coated untanned SRBC to a titer of 1:256. The same sensitized SRBC did not lyse when PBS was used in place of the anti-FP sera. This test indicated that factors on the FP 10000 sonicate preparation were immunogenic in rabbits, and that there were no reactive complement fixing antibodies in GPC' against FP 10000.

The reaction of various anticomplementary agents with normal GPC' was studied in tests that were incubated for 1 hr. at 37 C with GPC'. To the basic test system containing 0.1 ml GPC' and 0.8 ml VBD^{++} was added 0.1 ml of either FP 10000 at 1,250 µg/ml protein, FP 2000 at 4,100 µg/ml protein, inulin at 500 µg/ml dry weight, CoF at 10 anticomplementary units, WLPS extracted from FP cell walls at 100 and 300 µg/ml, and LPS at 100 µg/ml dry weight. The results are shown in Figure 1. The data is presented as the percent decrease in $C'H_{50}$ unit activity which is a function of the number of $C'H_{50}$ units consumed or missing in tests divided by the number of $C'H_{50}$ units present in VBD^{++} controls as determined by complement titration. Consumption of total complement was determined to be as follows: FP 2000, 73.3%; FP 10000, 34.0%; inulin, 61.7%; CoF, 70.7%; and 300 µg PLPS, 25.4%. Less active substances were 100 µg PLPS (14.9%), 300 µg WLPS (15.1%), 100 µg FP LPS (1.2%). The addition of an equal volume of heat treated GPC' to the test system failed to augment the anticomplementary activity of FP 2000 as shown by the reported 58.3% consumption (of available $C'H_{50}$ units). However, the addition of an equal volume of heat treated and SRBC absorbed anti-FP sera at a 1:2 dilution enhanced the consumption of GPC' by FP 2000 to a level greater than 85.8%.

The reactions of FP 2000 at 4,100 µg protein, inulin at 500 µg dry weight, and FP 2000 at 4,100 µg protein combined with 0.1 ml or a 1:2 dilution of heat treated and SRBC absorbed anti-FP sera were also tested with normal GPC' in an ice bath at 4 C. The results are shown in Figure 2 and reveal that incubation at 4 C was associated with a reduced mean level of

complement consumption by FP 2000 (30.8%) and inulin (20.5%).

The effect of extended incubation periods was evaluated in complement consumption assays conducted for 18 hr. at 4 C. Duplicate tests were run for each anticomplementary agent. One of the duplicates was incubated at 37 C for the first of the 18 hours. Inulin at 500 µg dry weight and FP 2000 at 4,100 µg protein alone and in conjunction with 0.1 ml of a 1:2 dilution of heat treated and SRBC absorbed anti-FP sera were tested with normal GPC' in the standard 1 ml complement consumption assay. The results are shown in Figure 3. As with the 1 hr. test at 4 C, the anticomplementary activity of FP 2000 and inulin was reduced at lower temperatures. However, when compared with total consumption in 1 hr at 4 C (30.8% and 20.5%) less complement was available for titration at the end of the 18 hr. test. Where the tests were incubated for the first hour at 37 C, FP 2000 and inulin consumed $C'H_{50}$ units of GPC' to levels of 74.3% and 77.9% respectively. The anticomplementary activity of FP 2000 in the presence of anti-FP sera was not affected by the cold temperatures as GPC' was consumed in excess of 85.0%.

After showing that sonicate preparations of F. polymorphum were anticomplementary to normal GPC', an attempt was made to establish the cellular location of the anticomplementary factors. FP 10000 at 1,925 µg protein, FP 2000 at 6,200 µg protein, and FP cell walls at 7,200 µg protein were utilized in the standard complement consumption assay. Inulin at 500 µg dry weight was used as a control. A duplicate set of tests was also performed utilizing the same preparations after treatment in a boiling water bath at 100 C for 30 min. These preparations were designated HFP. The results are shown in Figure 4. The unheated FP 10000, FP 2000, FP cell walls, and inulin reduced the titratable complement levels in GPC' by 71.0%, 71.0%, 69.7% and 71.0% respectively. Heat treatment of FP 2000 and FP cell walls did not affect the ability of these preparations to re-

duce complement activity while there was a reduction in the ability of heat treated FP 1000) to decomplement GP sera from 71.0% to 43.4%. The anticomplementary activities of several different bacterial preparations were compared. In each test, 500 µg/ml of one of the following preparations were used: (1) FP cell walls; (2) FP 2000 cell walls; (3) F. polymorphum acetone dried whole cells; (4) WLPS and PLPS from acetone dried whole F. polymorphum, and (5) a cell wall preparation Leptotrichia buccalis (ATCC # 19616) designated LB cell walls. The results of incubation with GPC' at 37 C for 1 hr. are shown in Figure 5. FP whole cells consumed complement at a rate of 49.6% as did the standardized FP cell walls. FP 2000 cell walls reduced the $C'H_{50}$ unit activity of GPC' by 55.4%, and the PLPS preparation extracted from acetone dried F. polymorphum whole cells consumed complement by 22.3%. LB cell walls were more active showing anticomplementary activity of 83.9%. WLPS displayed no anticomplementary activity.

An evaluation of the endotoxicity associated with different preparations of F. polymorphum and L. buccalis was accomplished using the Limulus amebocyte lysate. The results indicate that FP whole cells, FP cell walls, LB cell walls, FP WLPS, and FP PLPS all were capable of producing a positive reaction in the Limulus lysate assay at concentrations of 5 ng/ml.

DISCUSSION:

A complement consumption assay measured the consumption of GPC' in the presence of several different bacterial preparations of F. polymorphum and two other anticomplementary agents, inulin and CoF. Inulin and CoF were tested as possible controls for the alternate complement pathway (Osler and Sandberg 1973). Anti-FP was added to the FP 2000 sonicate in appropriate tests for activation of the classical pathway.

Initially, tests were performed at 37 C for 1 hr. which will support both alternate and classical pathway activity. It was shown in these tests (Fig. 1) that the various preparations of F. polymorphum, inulin, and CoF were capable of lowering the titratable C'H_{50} unit activity by different degrees. When used without anti-FP, FP 2000 showed greater anticomplementary activity than FP 10000, and this was probably due to either the higher protein concentration in FP 2000 or associated with the greater number and size of the cell wall fragments believed to be present in FP 2000. The suggestion that cell walls may be the source of anticomplementary factors in F. polymorphum is consistent with the reported anticomplementary activity of LPS which is a major component of the gram negative cell wall (Costerton, Ingram and Chang 1974). However, by comparison, purified WLPS and PLPS extracted from cell walls (Fig. 1) appeared to show lower anticomplementary activity than the cell walls. The concentrations of WLPS and PLPS (100 - 300 µg/ml) were chosen because others (Bladen et al. 1967; Gewurz et al. 1968) have reported that maximum complement consumption by phenol/water extracted LPS from V. alcalescens was achieved within a range of 100 µg and 300 µg.

To obtain a better index of the relative anticomplementary activity associated with several preparations of F. polymorphum, 500 µg was utilized as a standard concentration of each agent in the complement consumption

assay. The results indicated that 500 µg of FP whole cells, FP cell walls, and FP 2000 cell walls reduced the titer of $C'H_{50}$ units to essentially the same degree (Fig. 5). However, 500 µg of WLPS that had been extracted from acetone dried whole cells did not show any anticomplementary activity. Some consumption of $C'H_{50}$ unit activity was observed by PLPS extracted from the same acetone dried cells. The relative differences in anticomplementary activity between the phenol and water phase lipopolysaccharide preparations extracted from cell walls reported earlier (Fig. 1) were also seen in this test with phenol/water extracts from acetone dried whole cells. However, it would appear that the lipopolysaccharide preparations extracted from the water washed cell walls may be more anticomplementary. The apparent lower activity observed in the acetone dried cells might have been caused by partial "defatting" with the cold acetone treatment (Kristoffersen and Hofstad 1970). If so, this finding would be in support of studies which indicate that the fatty acid residues of the lipid A moiety may be responsible for the anticomplementary activity of LPS (Gewurz et al. 1968; Galanos et al. 1971). The finding that 500 µg of distilled water washed lyophilized whole FP cells showed the same activity as 500 µg of the water washed lyophilized cell walls is suggestive of inherent stability of anticomplementary factors to the methods used in cell wall preparation. Purification of WLPS to FP LPS by ultracentrifugation had no effect on its anticomplementary activity at 100 µg/ml.

From the extreme variability in complement consumption shown by different bacterial preparations of F. polymorphum, it would appear that these results may be a reflection of the manner in which each agent was prepared, the physical character of the preparation, and/or the heterogeneity of each of the preparations. Nowotny et al. (1966) have compared

the biologic activity of LPS prepared by phenol/water, trichloroacetic acid, and other extraction techniques and found that each LPS showed different biologic activity and serologic specificity. It was suggested by Nowotny (1963) that the biologic activity displayed by any of the LPS preparations did not necessarily indicate the potential of LPS in situ associated with the gram negative cell wall. These reports indicate that lipopolysaccharide preparations may be artifacts of laboratory extraction methods, and that their variability in endotoxic activity is probably due to the alteration or removal of essential reactive groups.

Phillips, Snyderman and Mergenhagen (1972) have presented evidence that a GPC' preparation might contain antibody that could react with LPS obtained from gram negative organisms. Hook, Snyderman and Mergenhagen (1970) found that both early and late acting complement components were consumed by LPS in hamster sera which suggested classical pathway activity in their tests. However, they were unable to demonstrate the same classical pathway activity in GPC'. In the data presented here, the amount of complement consumption by FP 2000 was not increased in the presence of an additional volume of heat treated GPC' (Fig. 1). This finding was an indication that the GPC' used in the complement consumption assay probably did not contain antibodies that would react with preparations of F. polymorphum which could augment the consumption of GPC' via the classical pathway. In addition, the cell controls in the hemolysis test showed that there were no complement fixing antibodies in the GPC'. The 1:10 dilution of GPC' in the hemolysis test was the same dilution employed in the complement consumption assay. We therefore suggest that reactive complement fixing antibodies were not involved in the consumption of GPC' by preparations of F. polymorphum. To show that there was potential for classical pathway

activity in the assay, anti-FP sera was added and all measurable $C'H_{50}$ units were consumed.

It has been established (Osler and Sandberg 1973) that alternate pathway consumption of complement is reduced in the cold. Using the complement consumption assay with incubation temperatures at 4 C, it was shown (Fig. 2) that the mean consumption of total complement activity by FP 2000 and inulin was decreased from 73.3% to 30.8% for FP 2000 and from 61.7% to 20.5% for inulin. The parallel reduction in anticomplementary activity of FP 2000 and inulin, the indicator for alternate pathway activity, suggests that FP 2000 consumed GPC' by utilizing reactions in the alternate pathway. In contrast, the classical pathway was probably not affected by the reduced temperature of incubation as shown by the greater than 85% reduction of $C'H_{50}$ unit activity by FP 2000 in the presence of anti-FP sera.

The observed effect of extended 18 hr. incubation at 4 C (Fig. 3) also supports the contention (Osler and Sandberg 1973) that the alternate pathway is adversely affected by the cold. Over the 18 hr. period at 4 C, there was progressive consumption of GPC' $C'H_{50}$ units by both FP 2000 and inulin so that by 18 hr. there had been a 58.6% and 44.1% reduction of complement activity respectively. But, these decreases were not as much as those observed at 37 C for 1 hr., 73.3% and 61.7% (Fig. 1), or when the first hour of the 18 hr. incubation period was maintained at 37 C, 74.3% and 77.9% (Fig. 3). Further analysis of the effect of temperature on the consumption of GPC' by FP 2000 and inulin indicated that at 37 C the maximum percent consumption had probably occurred by the end of 1 hr. and that continued incubation of the reaction mixture at 4 C for an additional 17 hr. produced very little, if any, additional consumption.

In addition to the fact that the mean consumption by FP 2000 in 1 hr.

was considerably lower in the cold than at 37 C, there was also a greater range of consumption data at 4 C after 1 hr. (26.6% vs. 5.2%) and after 18 hr. (47.6% vs. 2.8%). Both figures at 37 C (5.2% and 2.8%) are well within the sensitivity limits (±5%) of the complement consumption assay (Mayer 1971). The variation of data obtained in the cold is believed to be an expression of subtle differences in the amount of essential accessory serum factors in GPC' that were apparently not functionally significant at 37 C. It is projected that optimal interaction of anticomplementary factors and serum factors would eventually occur at 4 C if the time of incubation were extended sufficiently beyond 18 hr.

Based on our tests that indicated that the anticomplementary activity of F. polymorphum might be localized in cell wall fragments (Fig. 1), it was decided to test different bacterial preparations, each composed of a unique ratio of cytoplasmic to cell wall material. The heat stability of each preparation was also tested by vigorously heating the material in a boiling water bath for 30 min. The anticomplementary activity of F. polymorphum could be isolated in the cytoplasm free cell wall preparation, and the same activity was resistant to severe heat treatment (Fig. 4). Identical activity and heat resistance was observed with the FP 2000 preparation. The same ability to reduce the titratable $C'H_{50}$ unit activity was noted for the FP 10000 preparation, but heat treatment resulted in a marked reduction in its anticomplementary activity. These results indicated that the same apparent maximum anticomplementary activity seen with preparations containing cytoplasmic material (FP 2000 and FP 10000) could be isolated in FP cell walls. The larger cell wall fragments in FP cell walls and FP 2000 were probably important in maintaining a biologically active conformation of anticomplementary sites. The ability of FP cell walls and FP 2000 to consume complement was resistant to the denaturing

effects of heat while the active sites on the smaller cell wall fragments in FP 10000 were shown to be partially heat labile.

By comparing the anticomplementary reactions which occurred at 37 C and 4 C, and also considering the absence of reactive antibodies in the assay, it would appear that F. polymorphum is capable of alternate pathway activity in GPC'. The factors associated with this activity can be isolated in the cell walls of the organism in a heat resistant conformation. On a weight per assay basis, LPS extracted from the cell walls shows less potent anticomplementary activity. Therefore, it is suggested that similar cell wall preparations of this and other gram negative oral anaerobes could be used in further evaluations of their biologic activity in complement. In addition, this form of cytoplasm free cellular debris, rather than the laboratory phenol/water preparations of LPS, may have more significance in the proposed role of F. polymorphum in clinical periodontal disease.

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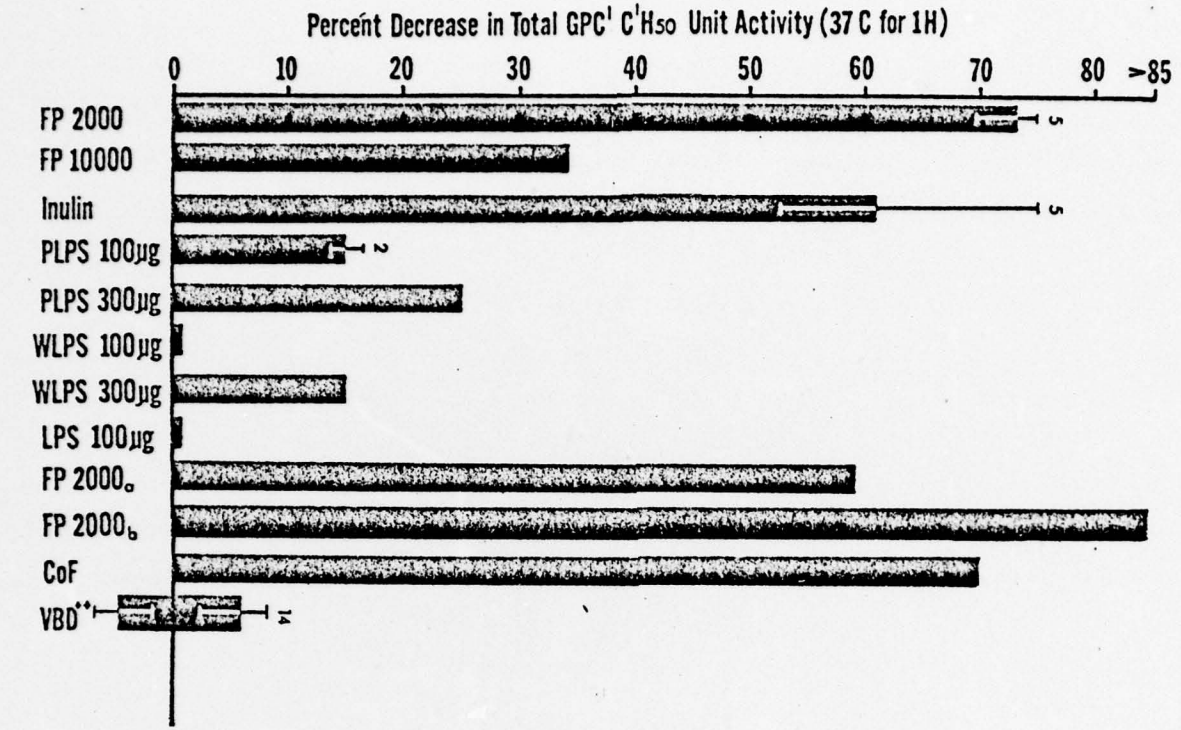
Percent Decrease in Total GPC' C'Hso Unit Activity (37 C for 1H)
0
10
20
30
40
50
60
70
80
>85
FP 2000
FP 10000
Inulin
PLPS 100µg
PLPS 300µg
WLPS 100µg
WLPS 300µg
LPS 100µg
FP 2000a
FP 2000b
CoF
VBD**
5
5
2
14

Figure 1. This bar graph shows the relative anticomplementary activity of several agents in the standard 1 hr. 37 C complement consumption assay. The agents used in the tests were as follows: FP 2000 at 4,100 µg, FP 10000 at 1,250 µg, inulin at 500 µg, CoF at 10 anticomplementary units, WLPS at 100 and 300 µg, and LPS at 100 µg. The vertical lines above and below the mean consumption indicate the range of data from the mean of the number of tests indicated.

a. FP 2000 anticomplementary activity with 0.1 ml of undiluted heat treated normal GPC' added to the standard assay.

b. FP 2000 anticomplementary activity in the presence of 0.1 ml of anti-FP sera, diluted 1:2, that had been SRBC absorbed and heat treated.

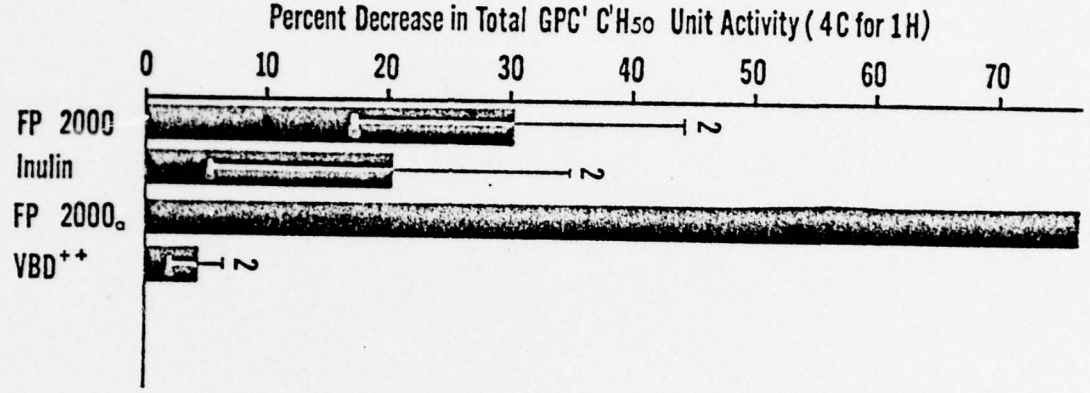
Percent Decrease in Total GPC' C'H50 Unit Activity (4C for 1H)
0
10
20
30
40
50
60
70
FP 2000
Inulin
FP 2000a
VBD++
2
2
2

Figure 2. This figure shows anticomplementary activities of FP 2000 and inulin at 4 C for 1 hr. FP 2000 was used in the test at 4,100 µg protein, and the inulin at 500 µg dry weight. The vertical lines above and below the mean indicate the range of data from the mean. The number of tests for each agent is shown.

a. FP 2000 anticomplementary activity that occurred with the addition of 0.1 ml of SRBC absorbed and heat treated anti-FP sera.

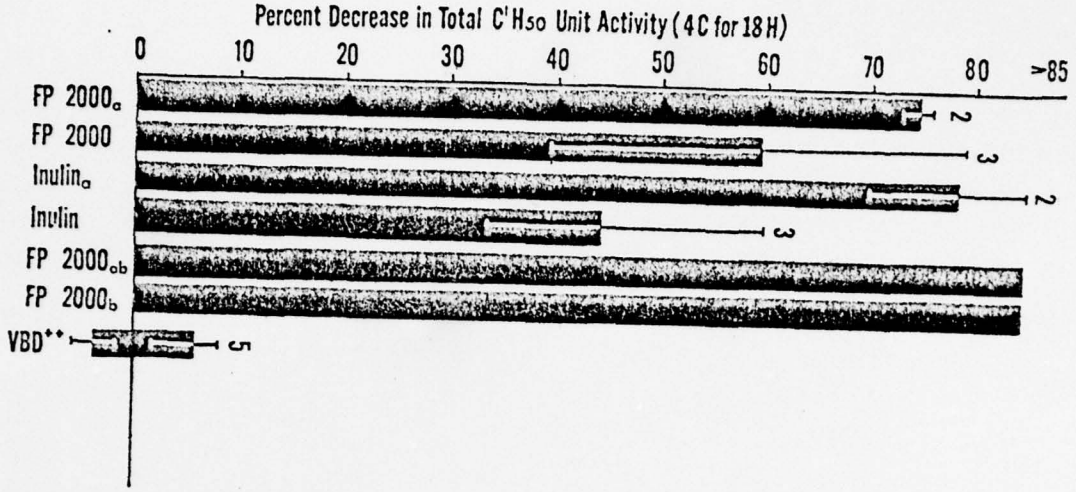
Percent Decrease in Total C'H50 Unit Activity (4C for 18H)
0
10
20
30
40
50
60
70
80
>85
FP 2000a
FP 2000
Inulina
Inulin
FP 2000ab
FP 2000b
VBD**
2
3
2
3
5

Figure 3. This figure shows anticomplementary activities at 4 C for 18 hr. of FP 2000 at 4,100 µg protein and inulin at 500 µg dry weight. Also, FP 2000 was tested in the presence of anti-FP sera. The vertical lines above and below the mean indicate the range of data from the mean. The number of tests for each agent is shown.

a. Tests where the first hour of incubation was at 37 C.

b. FP 2000 anticomplementary activity that occurred with the addition of 0.1 ml heat treated and SRBC absorbed anti-FP sera to the basic test.

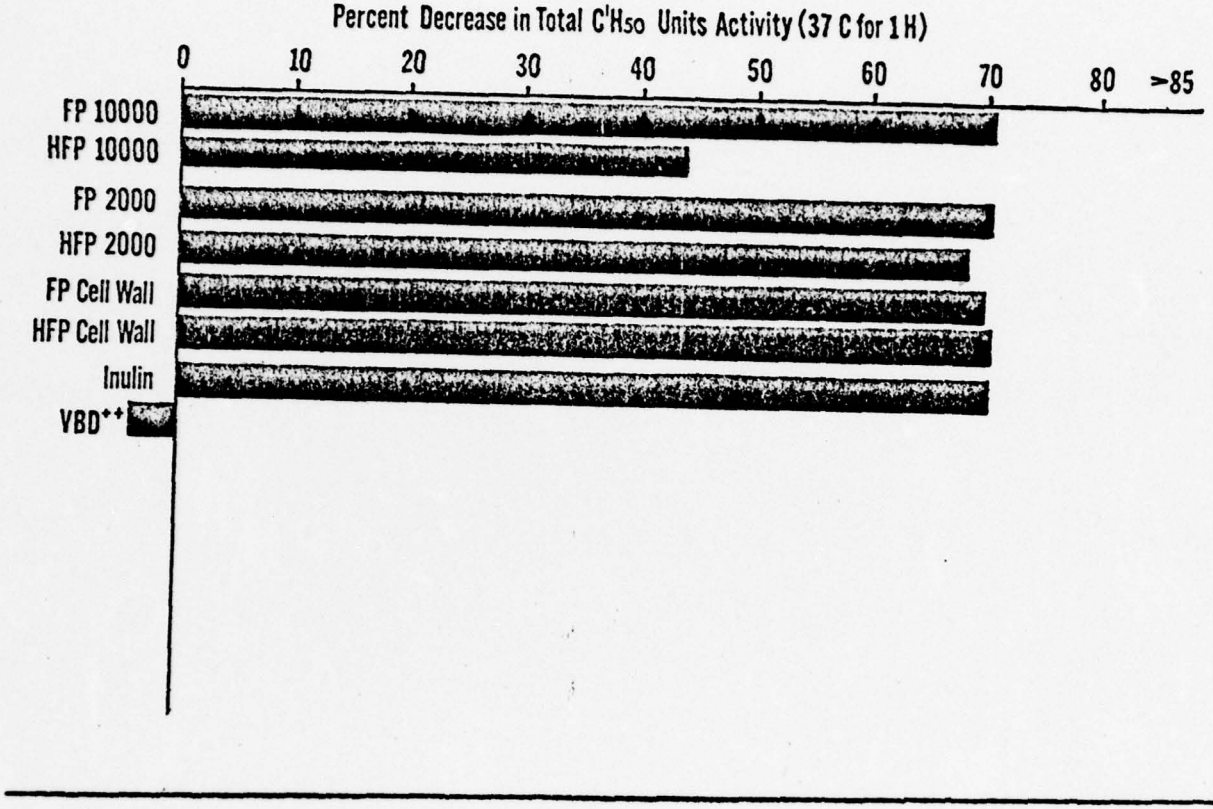
Percent Decrease in Total C'H50 Units Activity (37 C for 1 H)
0
10
20
30
40
50
60
70
80
>85
FP 10000
HFP 10000
FP 2000
HFP 2000
FP Cell Wall
HFP Cell Wall
Inulin
VBD++

Figure 4. This figure shows the anticomplementary reaction of different bacterial preparations of F. polymorphum in the standard complement consumption assay. These tests were performed using FP 10000 at 1,925 µg, FP 2000 at 6,200 µg, and FP cell walls at 7,200 µg protein.

HFP - FP preparations after treatment in a boiling water bath (100 C) for 30 min.

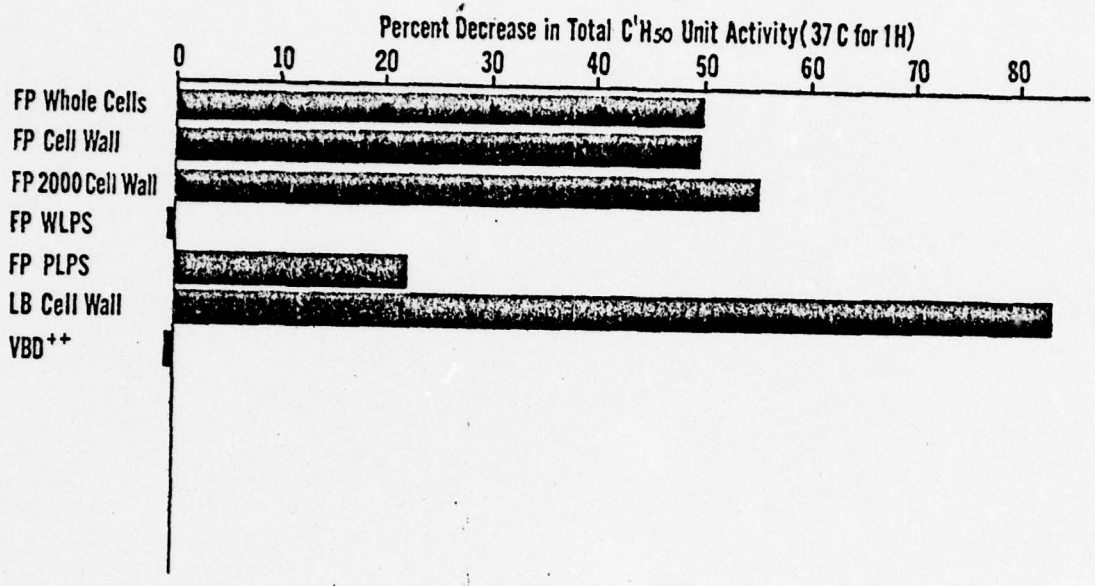
Percent Decrease in Total C'H50 Unit Activity (37 C for 1H)
0
10
20
30
40
50
60
70
80
FP Whole Cells
FP Cell Wall
FP 2000 Cell Wall
FP WLPS
FP PLPS
LB Cell Wall
VBD++

Figure 5. This figure shows the anticomplementary activity of 500 µg dry weight of each of the following preparations: FP whole cells, FP cell walls, FP 2000 cell walls, FP WLPS, FP PLPS, and LB cell walls.

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The materials and methods presented in this manuscript are basic to current mission oriented research at USAIDR, Washington, D.C. which will provide militarily relevant information on the epidemiology, etiology, treatment, prevention, and control of oral infections.

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