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ACTIVATION OF THE ALTERNATIVE PATHWAY OF COMPLEMENT IN HUMAN SERUM BY *Propionibacterium acnes* (*Corynebacterium parvum*) CELL FRACTIONS

GUY F. WEBSTER*, ULF R. NILSSON, and WILLIAM P. McARTHUR

Department of Pathology, School of Dental Medicine
University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract—Activation of the alternative pathway of complement is known to be initiated by bacterial structures. We have fractionated *Propionibacterium acnes* cells, purified various cell fractions, and tested their complement-activating ability in human serum chelated with ethyleneglycol bis-(β-aminoethylether)-*N*, N^1-tetraacetic acid. The majority of complement-activating activity was localized in the wall fraction. This activity was resistant to lipid extraction, protease, RNAse, DNAse and lysozyme treatment. $NaIO_4$, formamide, and hot (but not cold) trichloraacetic acid (TCA) extraction ablated the complement-activating capacity of cell walls. Compounds removed by extraction failed to consume significant hemolytic activity against antibody-coated sheep erythrocytes (EA). Addition of TCA-extracted soluble material to cell wall suspensions resulted in an inhibition of hemolytic consumption by the cell wall. These results indicate that, in *P. acnes*, complement-activating molecules are located in the cell wall and are carbohydrate in nature. Peptidoglycan, lipid, protein, and nucleic acids do not appear to contribute to the cell wall's ability to activate complement.

INTRODUCTION

Activation of the alternative pathway of complement (ACP) is important in the inflammatory response and in defense of the host against invading organisms (1). It is well established that in gram-negative bacteria, endotoxic lipopolysaccharide is responsible for ACP activation (2). In gram-positive bacteria, however, the active molecule(s) have yet to be identified for most organisms.

Propionibacterium acnes is a gram-positive anaerobic diptheroid found in high densities in human sebaceous areas (3). As a resident of the sebaceous follicle, *P. acnes* is the stimulus for the often disfiguring inflammation that

*Address for reprints—Department of Dermatology, School of Medicine, University of Pennsylvania, 36th & Hamilton Walk, Philadelphia, Pa. 19104.

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accompanies acne vulgaris. It has also been implicated in the pathogenesis of periodontal disease which, like acne, produces damage through inflammatory destruction of host tissues (4). Under the synonym of *Corynebacterium parvum*, *P. acnes* has shown to be a potent stimulator of antitumor defenses, presumably through the activation of macrophages (5, 6). We have previously shown that *P. acnes* cells are capable of activating human complement by both the classical and alternative pathways. The extent of classical pathways activation was found to be modulated by the anti-*P. acnes* antibody titer (7, 8). This study was undertaken in order to determine the structure(s) in *P. acnes* which activate the alternative pathway of complement.

MATERIALS AND METHODS

Bacteria. Propionibacterium acnes, ATCC 6919, was grown in prereduced peptone–yeast-glucose medium with added salts and Tween-80 for 4 days at 37°C under a 90% N_2–10% CO_2 atmosphere (9). Cells were fixed in formalin for 10 min, retrieved by centrifugation, and washed repeatedly with double-distilled water (ddw).

Preparation of Cell Fractions. Bacterial lipids were extracted using chloroform methanol by the method of Bligh and Dyer (10). Cells were then washed three times in acetone, suspended in ddw, and lyophilized. This procedure removes 99% of extractable lipid. It was noted that freezing in large volumes of ddw resulted in more easily suspended lyophilized preparations. These cells were then suspended in 10 ml ddw, 1 g of glass beads was added, and the slurry was then sonicated in a Branson sonifier at 90 W for 6–10-min periods, each separated by a 2-min cooling period. Throughout the sonification, cells were immersed in an ice bath. Sonic treatment for this period ruptured the overwhelming majority of cells, as determined by microscopic examination. After sonification, glass beads and intact cells were removed by centrifugation at 2000*g* for 10 min. Cell walls were then pelleted at 15,000*g* for 30 min. The supernatant from the final centrifugation was lyophilized and termed "cytosol fraction." After washing twice in ddw, the pellet was lyophilized and termed "crude cell wall" (CW) fraction. CCW was incubated for 4 h at 37°C with 50 μg/mg RNAse and DNAse (Sigma, St. Louis, Missouri) and then for 24 h with 50 μg/mg trypsin (Sigma); 0.1% NaN_3 was added to the incubation mixture to prevent microbial contamination. After multiple washing, this fraction was lyophilized and termed "partially pure cell wall" (PPCW).

In order to determine the class of molecule responsible for activation of the ACP, 100-mg quantities of PPCW were treated with various reagents. Digestion of peptidoglycan with lysozyme (Sigma) was attempted at 10, 0.1, and 0.01 μg/mg PPCW. Incubation was carried out at 37°C for 18 h. Ten percent TCA extraction was employed under two conditions: at 4°C on a rotator for 5 days and at 56°C for 90 min. Treatment with 0.05 M $NaIO_4$ (SIGMA) for 45 min at 37°C and extraction in formamide at 160°C for 20 min were also employed. After all treatments, cell walls were retrieved by 15,000*g* centrifugation and then washed exhaustively in ddw and lyophilized. The reaction mixture supernatants were dialyzed extensively against ddw at 4°C and lyophilized. The $NAIO_4$-, TCA-, and formamide-extracted materials were assayed for the presence of carbohydrate (11) and protein (12). No protein was detected in any soluble extract preparation. The extracts contained carbohydrate (approx. 900 μg/mg extract, expressed as glucose).

Activation of Complement. Activation of the ACP was selectively tested by incubation of serum and cell fractions in veronal buffer (13) which had been chelated with ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid and supplemented with 0.05 M Mg_2SO_4

(MgEGTA). This treatment removes free Ca^{2+} and selectively blocks the classical pathway by inactivating C1 (14). Each of the lyophilized fractions was suspended or dissolved in MgEGTA, and serial dilutions were performed. Equal volumes of normal human serum and dilution were mixed and incubated at 37°C for 30 min; activation of complement was stopped by plunging the reaction tubes into an ice bath at the end of the incubation. Total residual hemolytic activity was immediately assayed after the method of Mayer (13) and expressed as a percentage of the activity of serum incubated with buffer alone. *P. acnes* preparations failed to consume hemolytic activity when incubated in an ice bath rather than 37°C.

Immunoelectrophoresis (IEP) as previously described (7, 8) was used to confirm the inhibition of the classical pathway. Serum and serum treated with MgEGTA was incubated for 30 min at 37°C with 500 μg/ml PPCW, and then electrophoresed for 90 min at 90 V in Agarose (Seakem, Marine Colloids, Rockland, Maine) in high resolution buffer (Gelman, Ann Arbor, Michigan) with 0.01 M EDTA. Rabbit anti-human C4 was used to develop the gels (7, 8).

RESULTS

Effect of Lipid Extraction on ACP Activation. Whole cells consumed 100% total hemolytic activity at a concentration of 500 μg/ml. Extensive extraction of cellular lipid by chloroform-methanol and acetone failed to diminish or enhance the ability of whole cells to activate the ACP (Figure 1).

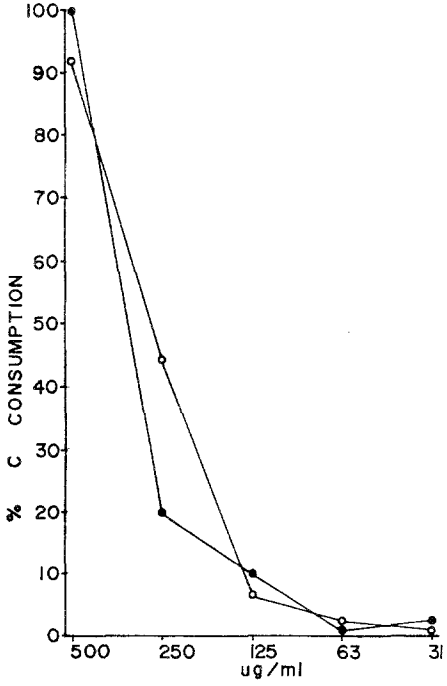

Fig. 1. Effect of lipid extraction on consumption of hemolytic activity by whole cells. o—o, extracted cells; •—•, unextracted cells.

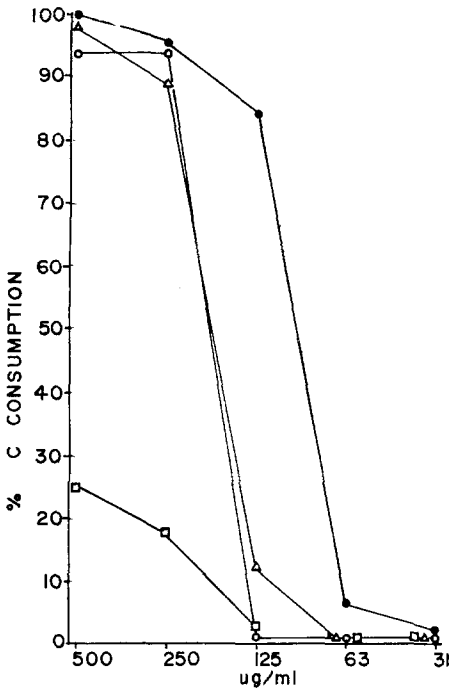

Fig. 2. Consumption of hemolytic activity by whole cells and cell fractions. •—•, PPCW; ○—○, CCW; △—△, cells; □—□, cytosol.

Comparison of Cell Fractions' Activity. As shown in Figure 2, all preparations consumed hemolytic activity in a dose-dependent manner. PPCW was the most potent activator, consuming 82% at a concentration of 125 μg/ml. CCW and whole cells had approximately the same activity, consuming 88 and 82%, respectively, at 250 μg/ml. Cytosol possessed minimal activity, consuming 24% at the highest dosage.

Effect of Lysozyme on PPCW. Lysozyme treatment at 10, 1, 0.1, and 0.01 μg/ml PPCW failed to affect the potency of PPCW, as shown in Figure 3.

Effect of Extraction of Polysaccharides. Extraction of PPCW with cold TCA resulted in a partial decrease in complement-consuming activity, reducing consumption from 100% to 31% at 125 μg/ml (Figure 4). The extracted material failed to consume hemolytic complement. Extraction with hot TCA caused a marked loss of PPCW activity, reducing complement consumption to less than 15% at the highest dosage. Hot TCA extracted material failed to consume hemolytic activity (Figure 4). Extraction

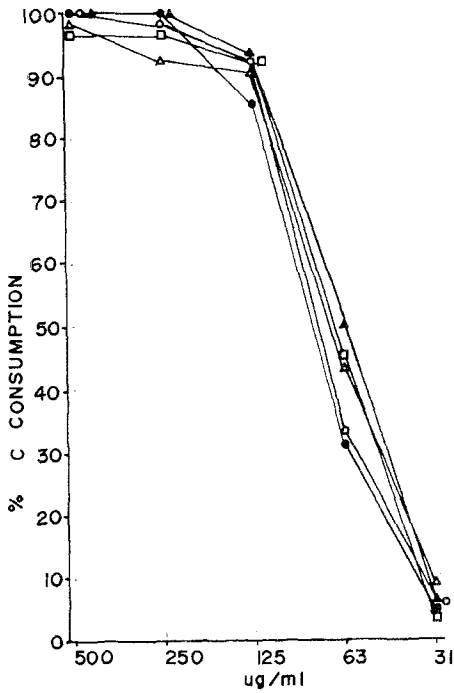

Fig. 3. Effect of lysozyme treatment on PPCW activity. ○—○, 10 μg/ml treated PPCW; △—△, 1 μg/mg treated PPCW; ▲—▲, 0.1 μg/mg treated PPCW; □—□, 0.01 μg/ml treated PPCW; ●—●, untreated PPCW.

with $NaIO_4$ had a similar effect to hot TCA extraction (Figure 5). Activation was less than 10% at 500 μg/ml for walls and 0 for the soluble extract. Removals of sugars from PPCW by the hot formamide technique also ablated the consuming activity of PPCW (Figure 6). The extracted material did not consume hemolytic activity.

Inhibition of Complement Consumption by Hot TCA Extracts. Addition of 1 or 2 mg/ml hot TCA extract to serial dilutions of PPCW produced a 96% inhibition of hemolytic complement consumption at 42 μg/ml PPCW and 24% inhibition at 83 μg/ml PPCW, respectively, when compared to PPCW dilutions to which buffer alone had been added (Figure 7). The 1:2 dilution of serum used in this assay did not diminish its lytic capacity in the assay system. The greater percentage consumption by PPCW in this system (as compared to Figures 2–6) may be due to the lower concentrations of serum used.

Immunoelectrophoresis. IEP of PPCW-treated serum revealed cleavage of C4. EDTA- and MgEGTA-treated sera failed to demonstrate C4

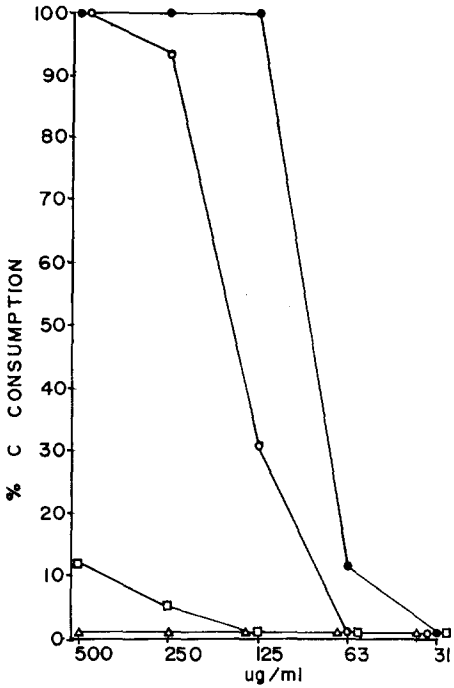

Fig. 4. Effect of TCA extraction of PPCW. •—•, PPCW; ○—○, cold TCA-extracted PPCW; □—□, hot TCA-extracted PPCW; △—△, hot TCA-soluble extract.

conversion, indicating that the MgEGTA treatment of serum was effective in inhibiting the classical pathway.

DISCUSSION

We have shown that exhaustive extraction of lipid does not affect the potency of whole *P. acnes* in activating the ACP. Sonic rupture of *P. acnes* yielded CCW and cytosol preparations. The CCW fraction contained the most potent complement activators. The cytosol fraction had very little activity, demonstrating only 23% consumption of hemolytic activity at 500 μg/ml. This minimal activity may be due to contamination with cell wall fragments which were not removed by centrifugation. Enzymatic digestion of RNA, DNA, and protein enhanced the potency of CCW, presumably by enriching the proportion of active molecules in the preparation. Thus the major complement activator in *P. acnes* resides in the cell wall and is neither protein, lipid, or nucleic acid in nature.

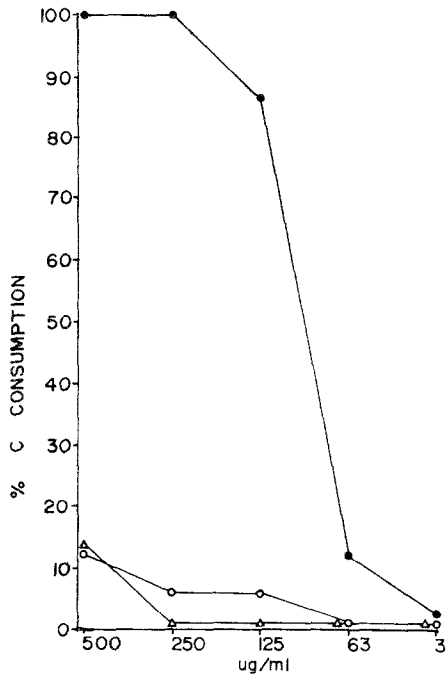

Fig. 5. Effect of $NaIO_4$ treatment on PPCW. •——•, PPCW; ○——○, $NaIO_4$-treated PPCW; △——△, $NaIO_4$-soluble extract.

Treatments which remove or alter cell-wall sugars (TCA, formamide, and $NaIO_4$ treatments) markedly reduced the capacity of the walls to activate complement. The extracted sugars all failed to consume significant hemolytic activity. Incubation of PPCW with lysozyme, an enzyme that degrades peptidoglycan, had no effect on the potency of the PPCW. This suggests that either the activator is resistant to lysozyme and, therefore, not peptidoglycan or that the *P. acnes* peptidoglycan is not attacked by lysozyme. No reduction in optical density of the PPCW suspension was evident after lysozyme treatment, favoring the latter explanation. The ability of TCA extracts to inhibit hemolytic consumption by PPCW may be evidence for the ACP-activating capacity of these preparations. This inhibitory activity can be interpreted to indicate that the extracts compete with the PPCW for early ACP components, rendering a portion of them unavailable for efficient, surface-bound conversion of C3-9.

Johnson and Cummins (15) have shown that *P. acnes* cell walls contain glucose, galactose, mannose, and galactosamine. These sugars are removed by hot TCA extraction (16) and might be expected to be present in our hot TCA extracts. The $NaIO_4$ and hot formamide extractions used have

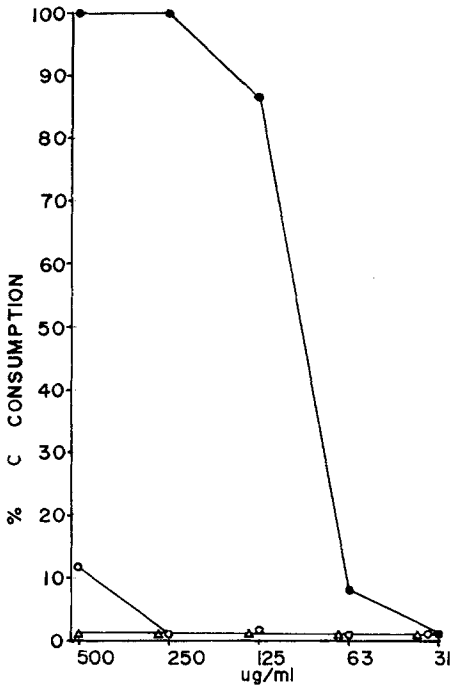

Fig. 6. Effect of formamide extraction of PPCW; •—•, PPCW; ○—○, formamide-extracted PPCW; △—△, formamide-soluble extract.

previously been shown to extract teichoic acids (if present) as well as polysaccharides from bacteria. Generally, teichoic acids are readily extracted by cold TCA treatment for several days. Hot TCA extraction is more vigorous and will remove teichoic acids as well as other sugars and may degrade them in the process (17). Our inability to completely remove the complement-consuming activity from PPCW with cold TCA extraction speaks against teichoic acids being the major activator in *P. acnes* cell walls. This is supported by the fact that *P. acnes* cell walls contain very little phosphorus (C. S. Cummins, personal communication), which is presumptive evidence for the scarcity of teichoic acids (17). Likewise, the inability of lipid extraction to diminish activation rules out lipoteichoic acids as the major complement activator. Thus, it appears that a non-teichoic acid polysaccharide, most likely containing glucose, galactose, mannose, and/or galactosamine may be the ACP activator in *P. acnes* cell walls. We have also shown that peptidoglycan prepared by the extraction of these sugars from PPCW possesses insignificant ability to consume complement.

Our finding that complement-activating activity in *P. acnes* is localized in the cell wall agrees with that of Okuda et al. (18) and is in concordance with the data for several other organisms (19–27) using similar techniques

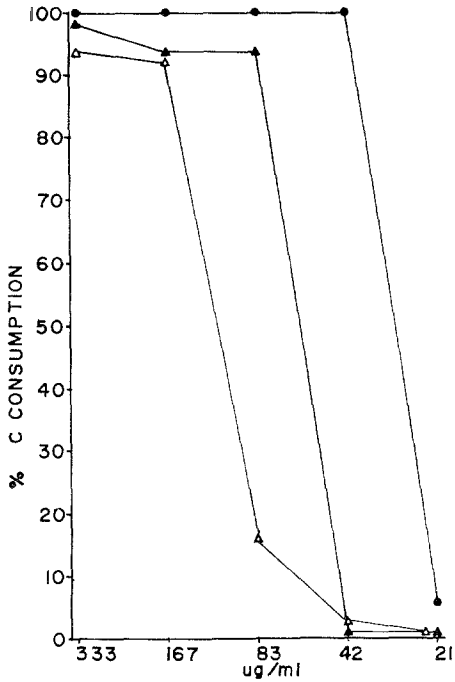

Fig. 7. Inhibition of hemolytic consumption by TCA-extracted material. •—•, PPCW, ▲—▲, PPCW + 1 mg/ml TCA extract; △—△, PPCW + 2 mg/ml TCA extract.

as used in this study, which identified teichoic acids as the pneumococcal alternative pathway activating molecule. As we have found in *P. acnes*, peptidoglycan from pneumococci (27), group A streptococci (28), and *Staphylococcus aureus* (29) were found to be inactive in complement consumption. Wilkinson et al. (25) found staphylococcal peptidoglycan to be far less active than teichoic acid in the consumption of complement. Similarly, cell wall defective L-phase variants were found to be potent activators of the ACP. The activator was removed by hot TCA extraction and to a lesser extent by cold TCA extraction (22). However, other studies have found that the complement activator in presumably different strains of group A streptococci to be peptidoglycan (20, 24) and membrane (29) and in *Micropolyspora faeni* to be peptidoglycan (23). The reason for this discrepancy may relate to differences in the strains tested or may relate to incomplete purification of peptidoglycan. We have found that methods which have been reported to yield purified peptidoglycan for other species are not sufficiently vigorous to extract all carbohydrates from *P. acnes* (unpublished observations). Thus, unless confirmed, "peptidoglycan" preparations may contain a significant amount of residual carbohydrate moities.

P. acnes (*C. parvum*) cells are potent stimulators of the reticuloendo-

thelial system (RES) and have been successfully used as immunopotentiators (5, 6). Cummins and Stimpson (30) have shown that the factor in *P. acnes* which causes stimulation is sensitive to hot, but not cold, TCA extraction and to treatment with 0.1 M $NaIO_4$. It is resistant to 2.5% sodium borohydride, protease digestion, and lipid extraction. Similarly, others have found the factor to be periodate sensitive (31, 32). Thus, the complement activator and the RES stimulator in *P. acnes* cells are chemically similar. A comparable relationship has been demonstrated for plant polysaccharides, whose anticomplementary activity has been shown to be proportional to their antitumor activity (33). Activation of factor B (34) and C3 (35) has been reported to activate macrophages in vitro, and preliminary data from this laboratory indicates that cobra venom factor depletion of complement inhibits the in vivo activation of murine macrophages by *C. parvum* (35).

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