INFECTION AND IMMUNITY, Apr. 1979, p. 233-243
0019-9567/79/04-0233/11$02.00/0

Vol. 24, No. 1

Interaction of Inflammatory Cells and Oral Microorganisms

VIII. Detection of Leukotoxic Activity of a Plaque-Derived Gram-Negative Microorganism

PIERRE BAEHNI,* CHI-CHENG TSAI, WILLIAM P. McARTHUR, BENJAMIN F. HAMMOND, AND NORTON S. TAICHMAN

University of Pennsylvania School of Dental Medicine and Center for Oral Health Research, Philadelphia, Pennsylvania 19104

Received for publication 3 January 1979

In the present study we identified a gram-negative anaerobic rod referred to as Y4 which was cytotoxic for human polymorphonuclear leukocytes. Y4 was isolated from dental plaque of a patient with juvenile periodontitis and presented most of the taxonomic characteristics of *Actinobacillus* species. Under experimental conditions, viable Y4 were cytotoxic for human peripheral blood polymorphonuclear leukocytes in serum-free cultures. Cytotoxicity was dependent on bacterial concentrations and was enhanced in the presence of a fresh or heat-inactivated (56°C, 30 min) autologous serum. Leukotoxicity was independent of phagocytosis. Y4 leukotoxic effect was abolished when bacteria were heat treated (56°C, 30 min) or when incubations were carried out at 4°C instead of at 37°C. The leukotoxicity was monitored by electron microscopy and biochemically by measuring lactate dehydrogenase indicator of cell viability. No cytotoxic effects of Y4 on human mononuclear cells, chicken fibroblasts, or mouse macrophages were detected under the conditions studied. Polymorphonuclear leukocytes may play an important role in the host defense against bacteria in periodontal disease. The cytotoxic effect of Y4 for polymorphonuclear leukocytes presented in this study is the first report of a direct offensive microbial vector in a plaque-derived microorganism and may prove to be relevant in the pathogenesis of juvenile periodontitis.

Periodontal diseases represent a group of inflammatory disorders of the tooth-supporting tissues. In the vast majority of instances, these diseases affect adults in the middle and later years of life and slowly lead to the destruction of the tooth-supporting structures. In contrast, juvenile periodontitis (JP) is a relatively uncommon form of periodontal disease found primarily in adolescents. The onset of this condition occurs during the circumpubertal period, progresses rapidly, and, within a few years, results in extensive loss of alveolar bone surrounding the affected teeth (3, 13). These and other differences suggest that the etiology and pathogenesis of JP may be different from that of the more prevalent forms of periodontal disease.

Although plaque microorganisms and/or plaque constituents are etiological agents in all forms of periodontal disease (21), it has been suggested that JP may be the result of specific microorganisms not relevant to other forms of periodontal diseases (15, 20). Newman and Socransky (14) have isolated several anaerobic gram-negative rods from the subgingival flora of JP patients. Intraoral monoinfection of gnotobiotic rats with these plaque isolates resulted in rapid alveolar bone loss (10; A. C. R. Crawford, S. S. Socransky, E. Smith, and R. Phillips, J. Dent. Res. **56**:B120, 1977; J. T. Irving, S. S. Socransky, M. G. Newman, and E. Savitt, J. Dent. Res. **55**:B257, 1976). This suggests that these microorganisms may be periodontal pathogens in JP, but the mechanisms by which these organisms mediate tissue destruction have not been elucidated.

Impaired host defense may also play a role in the susceptibility to JP. Recent findings indicate that there are abnormalities in chemotactic and phagocytic functions of peripheral blood polymorphonuclear leukocytes (PMNs) (5, 6), as well as the lymphocyte transformation response to autologous plaque and some gram-negative microorganisms (12) in JP. The chemotactic abnormalities are not only cellular but also have humoral manifestations (6). Therefore, JP may be associated with a host defense mechanism defect(s), and it appears that some microorganisms may be specific for this disease. However,

evidence for a direct mechanism or vector for bacterial virulence is still lacking. In the present communication, we report on a microorganism which has been identified as cytotoxic for PMNs (P. Baehni, C. C. Tsai, N. S. Taichman, and W. P. McArthur, J. Dent. Res. **57**:53, 1978). This microorganism, which was isolated from the gingival sulcus of a patient with JP, is a gram-negative anaerobic rod which fits most of the taxonomic criteria of the genus *Actinobacillus* and is referred to as Y4. It has been shown that Y4 has the potential to promote periodontal lesions in monoinfected rats (10). Considering that Y4 has adverse effects on PMNs and that PMNs are the predominant inflammatory cells in the gingival sulcus, the interaction of Y4 and PMNs may have crucial implications for the initiation and/or perpetuation of JP.

MATERIALS AND METHODS

Microorganism. The microorganism used in this study was originally isolated from the gingival sulcus of a patient with JP by S. S. Socransky and colleagues (Forsyth Dental Center, Boston, Mass.). The terminology employed to identify this microorganism (Y4) in the present report will be that initially used in Socransky's laboratory. Recently, this microorganism has been characterized more fully.

The organism (Y4) was a short, nonmotile, gram-negative anaerobic rod. It fermented glucose with acid but no gas. It also fermented fructose, xylose, maltose, and inulin but did not ferment dulcitol, mannitol, inositol, sorbitol, glycerol, raffinose, arabinose, or salicin. It was catalase negative but benzidine positive, did reduce nitrate, but did not produce H_2S or indole. Fermentation of sucrose and lactose were variable, but in no instance did the pH value of the culture fluid go below pH 5.9 after 5 days. The moles percent guanine plus cytosine of the deoxyribonucleic acid was 44. Antisera prepared against whole cells or sonic extracts of Y4 by the procedure of Hammond (9) showed clear cross-reactions with sonic extracts of *Actinobacillus actinomycetemcomitans* ATCC 29522 in Ouchterlony gel diffusion but no major cross-reactions with any of the other gram-negative isolates tested from the human oral cavity, including *Bacteroides ochraceus*, "Capnocytophaga" isolates, *Bacteroides melaninogenicus* subsp. *asacharolyticus*, *Eikenella corrodens*, *Vibrio sputorum*, and several other unidentified gram-negative anaerobes. Immunoelectropherograms of Y4 and *A. actinomycetemcomitans* indicated the presence of at least three common antigens in sonic extracts of each bacterium. The antigens appeared to have similar, if not identical, electrophoretic rates and gave reactions of identity in Ouchterlony plates. Three American Type Culture Collection strains of *A. actinomycetemcomitans*, 29522, 29523, and 29524, were tested for leukotoxic activity against human PMNs. Strain 29522, isolated from a mandibular abscess, and strain 29524, isolated from a chest aspirate, were found to be cytotoxic for human PMNs in a manner similar to that described in this paper for Y4. On the basis of these data, the published work on *A. actinomycetemcomitans* and the genus *Actinobacillus* (16), and other unpublished observations on end products of metabolism (S. S. Socransky, personal communication), Y4 is tentatively identified as an *Actinobacillus* species probably corresponding to *A. actinomycetemcomitans*.

Y4 were grown anaerobically (95% N_2 and 5% CO_2) in Brewer Anaerobic Jars from stock lyophilized cultures in thioglycolate broth (Difco Laboratories, Detroit, Mich.) for 48 h. Bacteria were harvested by centrifugation at 16,000 × *g* for 5 to 15 min at 4°C, washed in cold 0.9% saline solution, and suspended in Hanks balanced salt solution (HBSS) at appropriate concentrations determined by direct counting in Petroff-Hausser chambers.

Preparation of cells. Human peripheral blood PMNs were isolated from heparinized venous blood of healthy donors by dextran sedimentation (Dextran T500; Pharmacia Fine Chemicals, Piscataway, N. J.), followed by centrifugation on LSR (lymphocyte separation medium; Bionetics Laboratory Products, Kensington, Md.) to yield a preparation containing 90 to 95% PMNs (24). Cells were suspended at a concentration of 20×10^6 per ml of HBSS.

Human peripheral blood mononuclear cells were isolated from the same blood samples as the PMNs. Mononuclear cells were isolated from the top of the lymphocyte separation medium, washed in HBSS, and suspended to a concentration of 2×10^6 per ml of RPMI 1640 medium containing autologous serum (5%). Adherent monocytes were removed by incubation of the mononuclear cell suspension in petri dishes for 1 h at 37°C. Nonadherent (lymphocytes) cells were removed by gentle washing of the dishes with warm HBSS, concentrated by centrifugation, and resuspended (10^7 per ml) in HBSS.

Mouse peritoneal macrophages were obtained from peritoneal exudates from Swiss Webster mice which had been stimulated 4 days previously with 3 ml of thioglycolate broth injected intraperitoneally. Exudate cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in either 60-mm culture dishes or multiwell culture dishes at 37°C in an atmosphere of 5% CO_2 and 95% air. After 2 h of culture, the nonadherent cells were removed by sequential washing with warm HBSS, and the adherent cells were recultured for 48 h.

Chicken embryo fibroblasts were obtained from 10- to 11-day-old embryos which were trypsinized for 15 min in 0.25% trypsin at 37°C. The dispersed cells were washed in HBSS and cultured in flasks in Eagle minimal essential medium supplemented with fetal calf serum (10%, vol/vol), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2 and 95% air. Dead or nonadherent cells were removed by washing after 2 h of culture, and the adherent cells were reincubated. Upon reaching confluency (2 to 3 days), the embryo cells were removed from the flasks by a 1-min trypsinization and recultured in 60-mm culture dishes. Cells were grown in these dishes until they had reached a confluent monolayer and then were used in the experiments. Embryo cells at this stage had the morphology typical of cultured fibroblasts.

Incubation of cells with microorganisms. Duplicate cultures of PMNs (0.5 ml, 10×10^6 PMNs) were exposed to various concentrations of viable or heat-treated (56°C, 30 min) microorganisms (5, 25, 50, 100, and 200 bacteria per PMN) in a total volume of 1.0 ml of HBSS, using stoppered polypropylene centrifuge tubes (10 by 15 mm; Nalge Sybron Corp., Rochester, N.Y.) as culture vessels. Tubes were continuously agitated at 37°C in a gyratory water bath shaker (150 rpm; model F86; New Brunswick Scientific Co., New Brunswick, N.J.) for 5 to 60 min. Controls consisted of PMNs in HBSS without bacteria, PMNs in HBSS plus serum without microorganisms, and microorganisms alone or with serum in HBSS. At the conclusion of the experiment, cultures were placed on ice for 10 min and then centrifuged to remove PMNs and bacteria (24). In some experiments, culture conditions were varied by the inclusion of cytochalasin B (10 μg/ml) or iodoacetate (0.0001 M) in the incubation medium or by carrying the incubation out at 4°C instead of at 37°C. Culture supernatants were assayed for extracellular liberation of PMN constituents. In selected experiments, cells were collected for electron microscopy.

Human nonadherent peripheral blood cells (2×10^6 per ml, predominantly lymphocytes) were incubated in the absence of serum with Y4 (50 to 200 microorganisms per cell) for 1 h at 37°C in plastic culture tubes (13 by 100 mm). Mouse peritoneal macrophages in 60-mm petri dishes were exposed to viable Y4 (50 to 200 microorganisms per cell) in serum-free RPMI 1640 medium for 1 to 2 h at 37°C. Chicken embryo fibroblast monolayers in 60-mm petri dishes were exposed to 50 to 200 microorganisms per cell in serum-free RPMI 1640 medium for 1 to 2 h at 37°C. Both macrophage and fibroblast cell-to-Y4 ratios varied due to the slight variations in cell counts between culture dishes. Cell counts were done on cells removed from control cultures. Fibroblasts were removed by trypsinization (0.25%) for 2 min and the macrophages were removed by cold shock and scraping with a rubber policeman. Cells were counted in a hemocytometer.

Human sera. The effects of serum on PMN-microorganism interactions were evaluated in cultures to which 5.0% (vol/vol) fresh or heated (56°C, 30 min) serum was added. Autologous serum collected from the PMN donor was used. Sera were added to microorganisms immediately before the addition of PMNs.

Extracellular release of PMN lysosomal constituents. Lysozyme and myeloperoxidase served as representative lysosomal markers and were estimated in cell-free culture supernatants as described elsewhere (24). Results were expressed in one of two ways: as a percentage of the total available activity in the PMN culture or in terms of specific activity discharged into the cultures (24). In all calculations, the percentage or specific activity of products spontaneously released from control cultures (i.e., PMNs in HBSS alone, PMNs in HBSS plus serum alone, or Y4 alone) was subtracted from experimental values to provide net release of individual markers. Lysosome release from control cultures ranged from 2.0 to 8.0% of the total available activity in PMN suspensions.

Monitoring of cellular viability. As a reflection of cell viability, lactate dehydrogenase was monitored in culture supernatants of PMNs and mouse macrophage cultures by using the Worthington Assay Kit (26) (Statzyme LDH; Worthington Diagnostics, Freehold, N.J.). Results were expressed in percent lactate dehydrogenase release from stimulated cells when compared with maximal release from PMNs exposed to 0.05% Triton X-100.

Viability of human lymphocytes as well as PMNs was assessed by trypan blue exclusion. Cell preparations were exposed to the Y4 microorganisms for various periods of time. At the end of the incubation period, the cells were chilled and a trypan blue suspension was added (to a final concentration of 0.2%). Measurement of the numbers of cells taking up trypan blue (indication of dead cells) after 2 to 3 min was made by visualization of the cells in a hemocytometer. The number of cells taking up the dye (dead cells) was expressed as a percentage of the total cells present in the control cultures. The relative numbers of mouse macrophages and chicken embryo fibroblasts that became detached from the petri dishes during exposure to Y4 were also used as an indication of cellular alterations. The detached cells were removed, concentrated by centrifugation, and counted in a hemocytometer. The relative numbers of detached cells were expressed as a percentage of total cells in the culture. The viability of these cells was also examined by the trypan blue exclusion test. The detached cells were found to be nonviable by trypan blue exclusion.

Electron microscopy of PMN-microorganism interaction. PMN-microorganism interaction was evaluated with special attention made to the morphological changes in the PMN ultrastructure. PMNs were incubated at 37°C with viable or heat-treated Y4 (50 microorganisms per PMN) for 5 to 20 min in the presence or the absence of serum. At the conclusion of the experimental period, cell cultures were fixed in 1% osmic acid and processed for electron microscopy as previously described (1).

RESULTS

PMN lysosome and lactate dehydrogenase release in response to Y4. In the absence of serum, viable Y4 microorganisms provoked dose-dependent extracellular release of PMN lysozyme and myeloperoxidase, as well as the cytoplasmic enzyme lactate dehydrogenase (Fig. 1). Heat-treated (≥56°C, 15 min) Y4 did not trigger detectable lysosomal or lactate dehydrogenase release from PMNs at ratios as high as 200 Y4 microorganisms per PMN after a 60-min incubation period (Fig. 1). The increase in PMN lysosome and lactate dehydrogenase release in response to viable Y4 (5 to 200 bacteria per PMN) was directly related to the length (5 to 60 min) of the incubation period (data not shown). If the incubation of PMNs and Y4 was carried out at 2 to 4°C, no lysosomal constituents or lactate dehydrogenase activity was released into the culture supernatant (Table 1).

The addition of cytochalasin B (10 μg/ml) to the culture mixture of viable Y4 and PMNs did

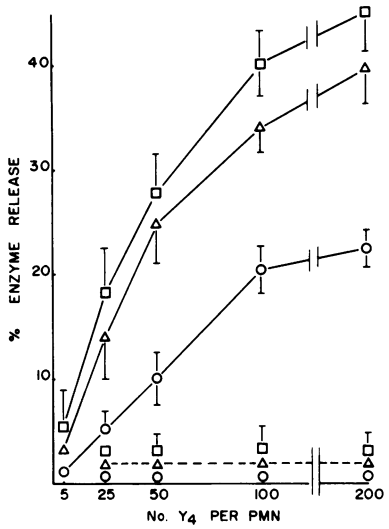


FIG. 1. *Release of lysozyme (□), myeloperoxidase (△), and lactate dehydrogenase (○) from PMNs exposed to various ratios of either viable Y4 (——) or heat-treated Y4 (- - -) for 1 h at 37°C in serum-free cultures. Results are expressed as the percentage of enzyme activity in the culture supernatants compared with the total activity available in the culture. Each point represents the mean ± standard deviation for five separate experiments.*

not inhibit lactate dehydrogenase or lysosomal release from PMNs into the culture supernatant (Table 1). In the presence of 10^{-4} M iodoacetate, the degree of lactate dehydrogenase or lysosome release was not significantly different from that detected in cultures without iodoacetate (Table 1).

Effects of serum on PMN lysosome and lactate dehydrogenase release in response to Y4. The addition of normal, fresh, or heat-inactivated (56°C, 30 min) serum (5%, vol/vol) autologous to the PMN donor enhanced the levels of lactate dehydrogenase and lysozyme released from PMNs in response to various concentrations (25 to 100 Y4 microorganisms per PMN) of viable Y4 (Figs. 2 and 3). However, the addition of both fresh and heated normal autologous sera into cultures of PMNs and heat-treated Y4 induced PMN lysozyme but no lactate dehydrogenase release (Fig. 2 and 4).

Electron microscopy of PMN-Y4 interactions. PMNs exposed to viable Y4 (50 Y4 microorganisms per PMN) at 37°C for 5 to 20 min were monitored by transmission electron microscopy. Phagocytosis of viable Y4 was not observed at any time during the incubation period. After 5 min of exposure to viable Y4, signs of PMN cell injury characterized by perinuclear spaces were noted (Fig. 5). After 10 min, some PMNs appeared irreversibly damaged, as suggested by various degrees of loss of cytoplasmic organelles, aggregation of the cytoplasmic matrix, and nuclear pyknosis (Fig. 6). A majority of the cultured PMNs exposed to viable Y4 for 20 min or longer had lost the characteristic PMN

TABLE 1. *Modulation of lactate dehydrogenase release from PMNs exposed to Y4*[a]

Culture conditions	Enzyme release (% of total activity)[b]	
	Lactate dehydrogenase	Lysozyme
37°C	23 ± 1	39 ± 4
4°C	3 ± 2	1 ± 1
Cytochalasin B[c]	18 ± 3	31 ± 3
Iodoacetate[d]	19 ± 2	36 ± 2

[a] Human PMNs were incubated with viable Y4 (200 microorganisms per PMN) for 60 min.

[b] Results expressed as the mean ± standard deviation of three or more determinations. Enzyme release expressed as a percentage of total available activity.

[c] Cytochalasin B, 10 μg/ml.

[d] Iodoacetate, 0.0001 M.

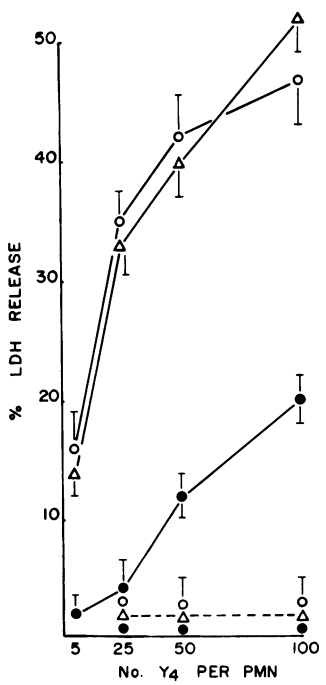


FIG. 2. *Lactate dehydrogenase (LDH) release from PMNs exposed to various concentrations of viable (●——●) and heat-treated (●----●) Y4 alone or Y4 in the presence of fresh (○) or heat-inactivated (56°C for 30 min) (△) autologous serum. Results are expressed as the percentage of enzyme activity in the culture supernatants compared with the total available enzyme activity in the cultures. Each point represents the mean ± standard deviation for five or more experiments.*

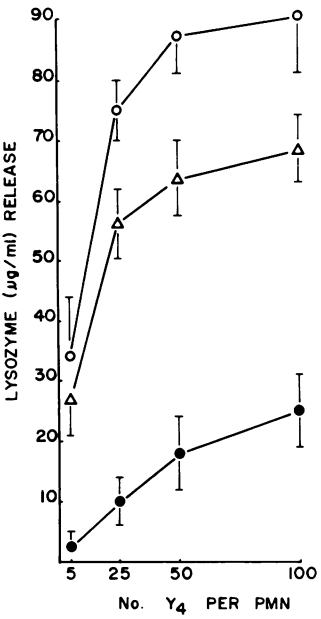


FIG. 3. *Effect of autologous serum (final concentration, 5%) on PMN lysozyme release in response to various ratios of viable Y4. PMNs were incubated with bacteria for 60 min at 37°C. Results are presented as mean ± standard deviation for three or more determinations. Symbols: ●, viable Y4 alone; ○, viable Y4 plus fresh autologous serum; △, viable Y4 plus heat-inactivated (56°C, 30 min) autologous serum.*

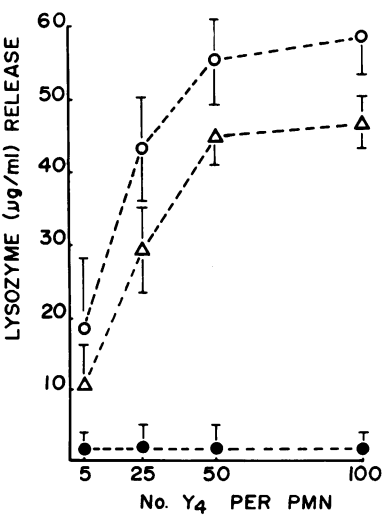


FIG. 4. *Effect of autologous serum (final concentration, 5%) on PMN lysozyme release in response to various ratios of heat-treated (56°C, 30 min) Y4. PMNs were incubated with bacteria for 60 min at 37°C. Results are presented as mean ± standard deviation for three or more determinations. Symbols: ●, heat-treated Y4 alone; ○, heat-treated Y4 plus fresh autologous serum; △, heat-treated Y4 plus heat-inactivated (56°C, 30 min) autologous serum.*

morphology and appeared to be dead or dying cells (Fig. 7). During the PMN exposure to Y4, gross discontinuities of the PMN plasma membrane were not seen until a stage of advanced cytoplasmic deterioration was apparent. PMNs exposed to heat-treated (56°C, 30 min) Y4 for 20 min demonstrated no phagocytosis or loss of lysosomal granules and did not show signs of cellular damage and deterioration noted in the PMNs exposed to viable Y4 (Fig. 8). The presence of either fresh or heat-inactivated normal serum in the viable Y4-PMN cultures did not alter the morphological changes of the PMN as compared with that seen in serum-free cultures of Y4 and PMNs (Fig. 9). PMNs exposed to heat-treated Y4 and serum showed no signs of cell breakdown, but phagocytosis of Y4 microorganisms was evident (Fig. 10). The morphology of erythrocytes and lymphocytes which were occasionally seen as contaminants of the PMN cell suspension were not changed as compared with controls by exposure to Y4 for 20 min.

Specificity of Y4 cytotoxicity. Under conditions similar to those in which Y4 were cytotoxic to PMNs, no significant cytotoxic effects on human mononuclear cells, chicken fibroblasts, or mouse macrophages were observed (Table 2).

DISCUSSION

The present paper reports on a cytotoxic effect on human cells of a microorganism which was originally isolated from dental plaque in a patient with JP. In these in vitro studies, this microorganism with the characteristics of the genus *Actinobacillus* and referred to as Y4 induced the release of lactate dehydrogenase, a cytoplasmic enzyme, from human PMNs. Release of lactate dehydrogenase was considered to be a marker of cell death (25) as well as tissue destruction (11). PMN cell death indicated by lactate dehydrogenase release was supported by direct electron microscopic observation of PMNs at various times during culture with viable Y4 microorganisms. At the ultrastructural level, obvious morphological changes after 20 min of incubation were observed. As early as 5 min after exposure to Y4 microorgansms, early ultrastructural alterations consisted of perinuclear spaces. Within 10 min of culture, some PMNs appeared to have various degrees of cytoplasmic and nuclear alterations. By 20 min, the majority of the PMNs had lost all of the characteristics of PMN morphology; the cytoplasm was completely disorganized and was accompanied by a total loss of lysosomal granules as well as other cytoplasmic organelles. Consequently, PMN cell death in response to viable Y4 microorganisms in serum-free cultures was

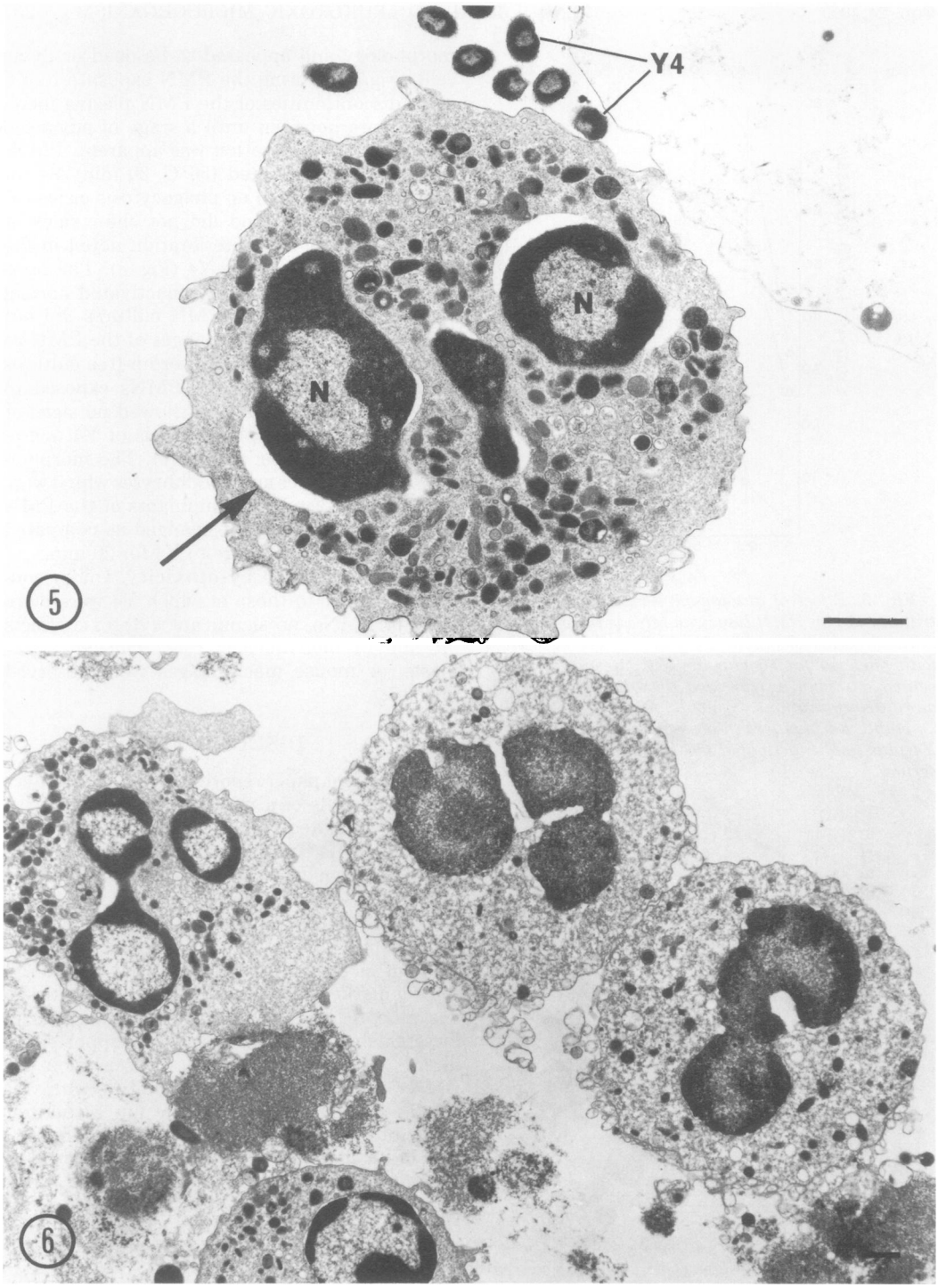


FIG. 5. *Electron micrograph of human PMNs incubated with viable Y4 for 5 min at 37°C in a serum-free culture. Bacteria were in close proximity to the PMNs, but no phagocytosis was observed. Although PMN cytoplasmic granules were generally well preserved, the perinuclear spaces (arrow) in the PMN suggested an early stage of cellular injury. N, Nucleus. Bar = 1 μm.*

FIG. 6. *PMNs exposed to viable Y4 for 10 min. This electron micrograph depicts various stages of PMN injuries: loss of cytoplasmic organelles, nuclear pyknosis, and aggregation of the cytoplasmic matrix. Bar = 1 μm.*

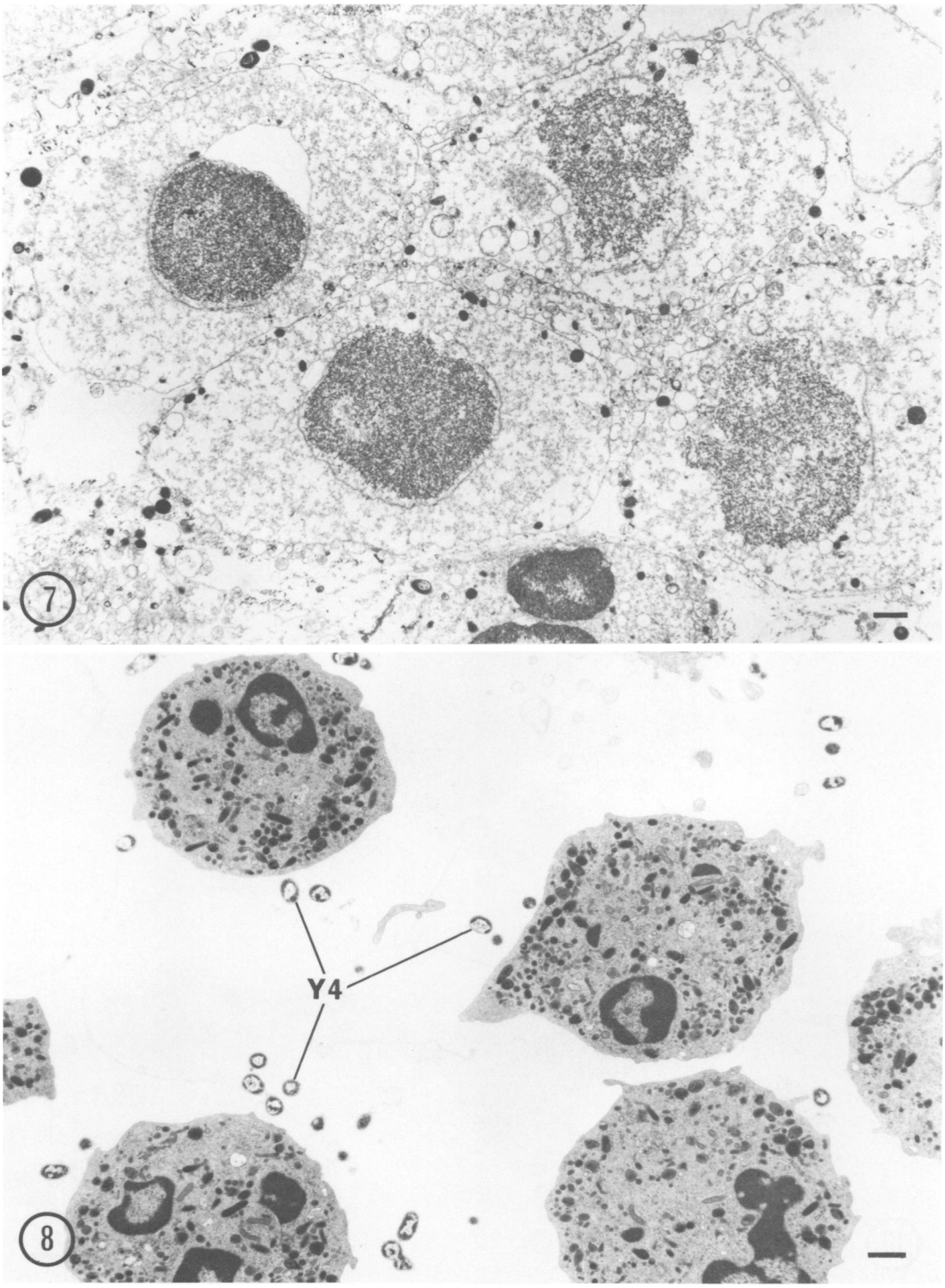
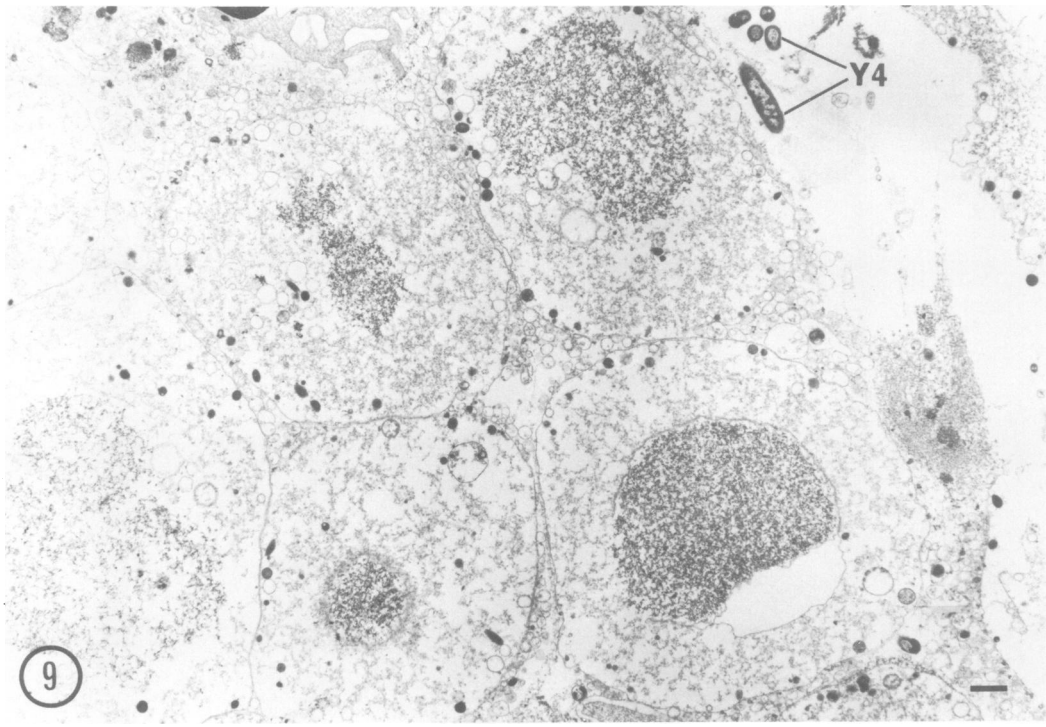


FIG. 7. *Electron micrograph of PMNs exposed to viable Y4 for 20 min in a serum-free culture. PMN injury appeared irreversible and was characterized by a complete loss of cellular organization in the majority of the cells. PMNs appeared swollen and had lost the morphological characteristics of polymorphonuclear cells. However, cytoplasmic membrane appeared generally intact. Bar = 1 μm.*

FIG. 8. *Electron micrograph of PMNs exposed to heat-treated (56°C, 30 min) Y4 for 20 min in a serum-free medium. PMNs showed no sign of cellular injury. Nuclei and cytoplasmic organelles, including lysosomes, appeared well preserved. Bacteria could be identified in the PMN proximity, but no phagocytosis was noticed. Bar = 1 μm.*



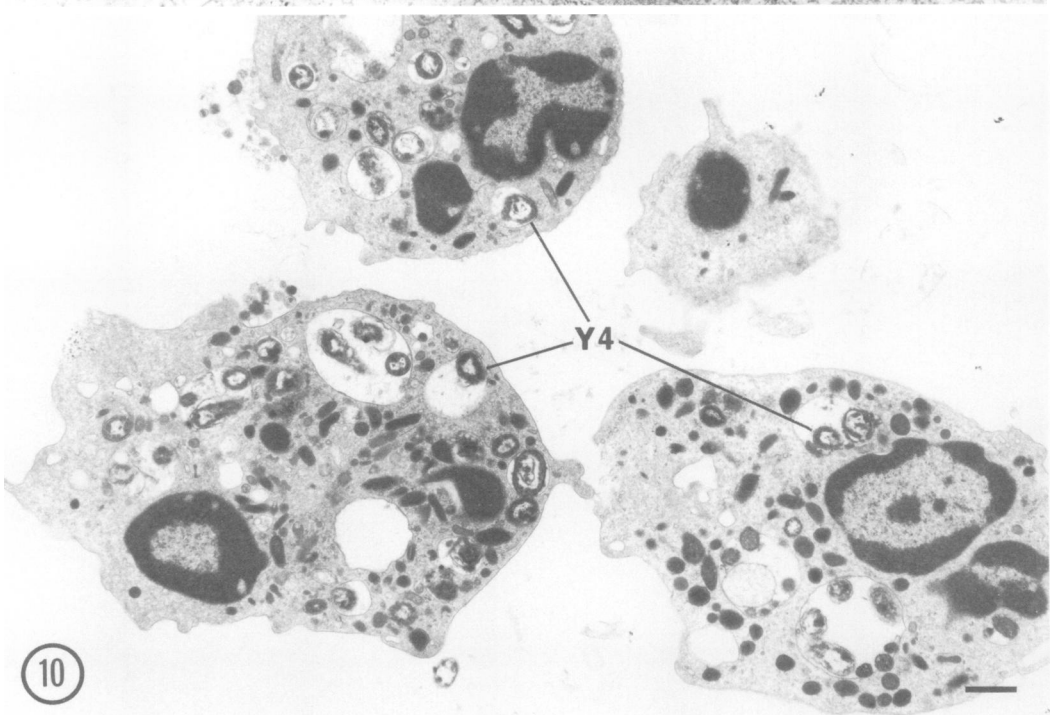


FIG. 9. *PMNs were incubated with viable Y4 for 20 min in the presence of fresh autologous serum. All PMNs appeared irreversibly damaged and showed similar morphological cellular alterations as observed in Fig. 7. Bar = 1 μm.*

FIG. 10. *PMNs were incubated with heat-treated (56°C, 30 min) Y4 for 20 min in the presence of fresh autologous serum. Bacteria could be identified within phagocytic vacuoles, and relatively fewer lysosomal granules remained in the cytoplasm of the PMNs compared with controls. PMNs did not exhibit any sign of cellular injury. Bar = 1 μm.*

TABLE 2. *Cytotoxicity of viable Y4 for various cells in vitro*[a]

Y4/PMN ratio	Dead cells ± SD (%)[b]			
	Human PMNs	Human mononuclear cells	Chicken fibroblasts[c]	Mouse macrophages[c]
0	5 ± 3	4 ± 4	3 ± 2	10 ± 6
50	85 ± 6	6 ± 3	5 ± 3	11 ± 3
100	95 ± 3	10 ± 5	7 ± 3	12 ± 4
200	96 ± 3	12 ± 4	6 ± 4	15 ± 8

[a] Cells exposed to various ratios of viable Y4 for 1 h at 37°C in serum-free medium.

[b] Dead cells were determined by their inability to exclude 0.2% trypan blue. The percentage was determined from the ratio: (stained cells/total cells) × 100. The dead cells in chicken fibroblasts and mouse macrophages were determined by counting the cells that became detached during exposure to viable Y4 and dividing these numbers by the total number of cells in control cultures. Total cells in control cultures were determined by removing them with trypsin (fibroblasts) or cold shock (macrophages) and counting them in a hemocytometer. More than 95% of the cells that became detached were found not to exclude dye, whereas more than 90% of the control cells did exclude dye and were considered viable. SD, Standard deviation of three or more determinations.

[c] Lactate dehydrogenase activities in these cultures exposed to Y4 were not greater than those in the control cultures.

detected by both electron microscopic observations and lactate dehydrogenase release.

In previous studies dealing with the PMN response to whole dental plaque (2, 24), viable plaque isolates (23, 25a) and microbial constituents (22), we had found that PMNs responded to certain of these stimuli by actively releasing lysosomal constituents. Upon interactions with these various stimuli, PMNs did not release detectable levels of lactate dehydrogenase or acquire the appearance of a dead or dying cell detectable by electron microscopy. The lack of lactate dehydrogenase release indicated that lysosomal release from PMNs was not associated with PMN cell death. Therefore, under the conditions tested, these stimuli were not toxic for PMNs. In these series of experiments, we reported also that a number of gram-negative plaque-derived microorganisms, including Y4, did not induce lactate dehydrogenase release from human PMNs in vitro (25a). The reason for the lack of toxicity of Y4 for PMNs in the previous experiments is not understood. However, the bacterial growth culture conditions were different, as was the stock culture from which Y4 was grown. In those previous experiments, Y4 was grown anaerobically in Trypticase soy agar; in the present report the microorganisms were grown in thioglycolate broth in an atmosphere of 95% N_2 and 5% CO_2. The original stock culture of Y4 obtained from S. S. Socransky was used as the source of Y4, whereas a descendent culture of the original was used in our previous work. Cultivation of microorganisms in synthetic medium in vitro has been shown to result in loss of toxic activity in other bacteria (18). Evidence suggests that reculturing of Y4 in vitro may also result in the loss of leukotoxic activity (unpublished observation).

A previous report on the induction of disease in gnotobiotic rats by gram-negative anaerobic plaque microorganisms from JP patients described Y4 as being flagellated (10). The organism used in this study was nonmotile but did produce disease as a monocontaminant in gnotobiotic rats (Socransky, personal communication). The reason for discrepancies in the description of this bacterium is not known. Possibly, it is due to morphological changes occurring in in vitro culturing. However, there seems to be little doubt that the organism Y4 used in these studies was a member of the genus *Actinobacillus*. Y4 was similar to *A. actinomycetemcomitans* ATCC 29522 in standard biochemical tests as well as antigenically. In addition, *A. actinomycetemcomitans* ATCC 29522 and 29524 were found to have leukotoxic activity against human PMNs similar to that described for Y4 (unpublished observations).

The nontoxic activity of whole plaque was somewhat surprising considering the diversity of the plaque composition and the discovery of the leukotoxic property of Y4. The pools of whole plaque tested previously were collected exclusively from patients presenting various degrees of chronic periodontitis. Because Y4 microorganisms were isolated from patients with JP, they might not have been included in these plaques. The possible absence of Y4 organisms from periodontitis plaque is supported by the fact that JP presents a different clinical condition from the chronic periodontitis (3, 13), and there is evidence that the microbial etiology may be quite different (15, 20).

The cytotoxic effect on PMNs of viable Y4 microorganisms in vitro seemed to be independent of phagocytosis, as indicated by electron microscopy. In addition, cytochalasin B, which is known to block phagocytosis in this system, did not significantly inhibit lactate dehydrogenase release from PMNs in response to Y4 alone or in the presence of fresh or heated serum. The lack of phagocytosis of Y4 by PMNs suggested that soluble bacterial products may be liberated from Y4. However, Y4 culture growth supernatants, as well as supernatants of 6-h cultures of washed viable Y4, did not contain detectable

cytotoxic activity of PMNs (unpublished data), suggesting that the leukotoxic activity of Y4 may not be characteristic of a traditional exotoxin.

Although the cytotoxic mechanism(s) of Y4 is not identified, certain characteristics of this cytotoxic activity have been defined. At 2 to 4°C, viable Y4 microorganisms were not cytotoxic to PMNs, as determined by lactate dehydrogenase release and electron microscopy. Heat treatment (56°C, 30 min) inactivated the cytotoxicity of whole Y4. The cytotoxicity of Y4 for PMNs also did not appear to require metabolically active PMNs since inclusion of 10^{-4} M iodoacetate to the culture medium did not alter the level of lactate dehydrogenase released. We have shown previously that iodoacetate inhibits PMN lysosomal release in response to a variety of stimuli (2, 24). In the present study, viable Y4, but not heated Y4, triggered both lactate dehydrogenase and lysosomal release, suggesting that lysosomal extrusion was the result of cell death.

The cytotoxic effect of viable Y4 was tested on other cells. Y4 did not seem to be toxic to human lymphocytes, mouse macrophages, and chicken fibroblasts in vitro under the conditions used. In addition, contaminating human erythrocytes in the PMN cultures were not lysed by viable Y4. However, under similar conditions (time, temperature, and serum-free medium), the same preparations of Y4 were cytotoxic for PMNs. Consequently, these data suggest that the Y4 cytotoxic activity may be a leukocidin, although more extensive studies need to be done to establish this as fact. Leukocidins have been identified in other bacterial species such as *Pseudomonas* (17), *Staphylococcus* (8), and *Streptococcus* (4). Some necrotic lesions produced by *Pseudomonas aeruginosa*, for example, are characterized by limited exudation and local tissue neutropenia (7). These observations are of interest when considered in the context of JP Studies of Y4-monocontaminated animals have shown mild inflammatory cellular infiltration in the gingival area (10). Similarly, patients with JP usually present little inflammation of the gingival tissues (3). The Y4 toxic activity appeared to be a leukocidin analogous to the leukocidins of strains of *Staphylococcus* (8) and *Pseudomonas* (18, 19) since it destroyed leukocytes (PMNs) without causing hemolysis. The Y4 leukocidin also did not correspond with the classical definition of an exotoxin since it was not released spontaneously in its growth culture. Heat sensitivity, as well as blocking of the cytotoxic activity of Y4 by the addition of trypan blue (unpublished data), suggested that an endotoxin was not involved. However, other similarities in the composition and the mode of action between Y4 leukotoxin and that described in other bacteria still remain to be determined.

Addition of both fresh and heat-inactivated normal autologous sera to viable Y4-PMN cultures resulted in enhanced lactate dehydrogenase release compared with serum-free cultures. In the presence of serum, heat-treated Y4 induced lysozyme release but not lactate dehydrogenase extrusion. Electron microscopic observations revealed that, with serum, heat-inactivated Y4 were phagocytosed, thus inducing PMN degranulation. The mechanisms for the enhancement of the toxicity of viable Y4 are still undefined. However, it may appear to be independent of antibodies since these sera were from young donors without history of JP. Preliminary studies using specific rabbit anti-Y4 serum indicated that antibodies have an inhibitory effect on Y4 cytotoxicity on PMNs. Complement does not seem to play a role, since heat-inactivated serum was as potent as fresh serum in enhancing the cytotoxic activity.

The microorganism used in this study was isolated originally from a patient with JP. This disease is characterized by a rapid alveolar bone loss in young adults accompanied with minimal inflammation of the gingival tissues and little plaque accumulation in the sulcus. The pathogenic mechanisms of JP are still unclear. Some investigators have found a PMN chemotactic defect, which suggests that the host defense is impaired in the patients (5, 6). Our study indicates that a microorganism associated with the disease may also play a role in suppressing the PMN host response. Together, these two observations may result in severe depression of local host defense in the sulcular region.

This is the first report of a direct pathological vector contained in a microorganism isolated from dental plaque. Although Y4 and similar organisms are found in high frequency in patients with JP, complete information concerning the distribution of this microorganism in plaque from patients with various types of periodontal disease and its taxonomy are unfortunately still lacking. Identification of the leukocidin activity in Y4 undoubtedly will prompt further work on the characteristics, the distribution, and the occurrence of Y4 and similar microorganisms from plaque.

ACKNOWLEDGMENTS

We gratefully acknowledge Jerry Cohen, Kathy Derr, Patrice Gibbons, Ann Stephenson, and Rollande Tremblay for their competent technical assistance, Bea McCray for her help in preparation of the manuscript, and Max Listgarten for the use of his electron microscope facility.

This investigation was supported by Public Health Service grants DE 02623 and DE 03995 from the National Institute of Dental Research and the Center for Oral Health Research. W.P.M. is a recipient of Research Career Development Award DE 00070 from the National Institute of Dental Research.

rus cytopathology, p. 466–486. *In* S. Mudd (ed.), Infectious agents and host defenses. Saunders, New York.
9. **Goldman, R.** 1976. The effect of cytochalasin B and colchicine on concanavalin A induced vacuolation in mouse peritoneal macrophages. Exp. Cell Res. **99**:385–394.
10. **Griffin, F. M. Jr., and S. C. Silverstein.** 1974. Segmental response of the macrophage plasma membrane to a phagocytic stimulus. J. Exp. Med. **139**:323–336.
11. **Gunther, G. R., J. L. Wang, I. Yahara, B. A. Cunningham, and G. M. Edelman.** 1973. Concanavalin A derivatives with altered biological activities. Proc. Natl. Acad. Sci. U.S.A. **70**:1012–1016.
12. **Hirsch, J. F., and B. Strauss.** 1964. Studies on heat-labile opsonin in rabbit serum. J. Immunol. **92**:145–154.
13. **Jones, R. B., J. C. Newland, and T. M. Buchanan.** 1978. Opsonization of *Neisseria gonorrhoeae*: role of antipili antibody. Clin. Res. **26**:397A.
14. **Mudd, S., M. McCutcheon, and B. Lucke.** 1934. Phagocytosis. Physiol. Rev. **14**:210–275.
15. **Ofek, I., E. H. Beachey, and A. L. Bisno.** 1974. Resistance of *Neisseria gonorrhoeae* to phagocytosis: relationship to colonial morphology and surface pili. J. Infect. Dis. **129**:310–316.
16. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature (London) **265**:624–625.
17. **Old, D. C.** 1972. Inhibition of the interaction between fimbrial hemagglutinins and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. **71**: 149–157.
18. **Oliver, J. M., T. E. Ukena, and R. D. Berlin.** 1974. Effects of phagocytosis and colchicine binding on the distribution of lectin-binding sites on cell surfaces. Proc. Natl. Acad. Sci. U.S.A. **71**:394–398.
19. **Rabinovitch, M.** 1968. Phagocytosis: the engulfment stage. Semin. Hematol. **5**:134–155.
20. **Salit, I. E., and E. C. Gotschlich.** 1977. Type I *Escherichia coli* pili: characterization of binding to monkey kidney cells. J. Exp. Med. **146**:1182–1194.
21. **Sharon, N., and H. Lis.** 1972. Lectins: cell-agglutinating and sugar-specific proteins. Science **177**:949–959.
22. **Shedder, W. I. H.** 1962. Fimbriae and haemagglutinating activity in strains of *Proteus hauseri*. J. Gen. Microbiol. **28**:1–7.
23. **Silverblatt, F. J.** 1974. Host-parasite interaction in the rat renal pelvis. A possible role for pili in the pathogenesis of pyelonephritis. J. Exp. Med. **140**:1696–1711.
24. **Silverblatt, F. J., and I. Ofek.** 1978. Influence of pili on the virulence of *Proteus mirabilis* in experimental hematogenous pyelonephritis. J. Infect. Dis. **138**:664–667.
25. **Silverblatt, F. J., and I. Ofek.** 1978. Influence of pili on the virulence of *Proteus mirabilis* in experimental pyelonephritis, p. 49–59. *In* E. Kass and W. Brumfitt (ed.), Infection of the urinary tract. Proceedings of the Third International Symposium on Pyelonephritis, London. Chicago University Press, Chicago.
26. **Silverstein, S. C., R. M. Steiman, and Z. A. Cohn.** 1977. Endocytosis. Annu. Rev. Biochem. **46**:669–722.
27. **Stossel, T. P.** 1978. How do phagocytes eat? Ann. Intern. Med. **89**:398–402.
28. **Stossel, T. P., R. J. Mason, T. Pollard, and M. Vaughan.** 1972. Isolation and properties of phagocytic vesicles. II. Alveolar macrophages. J. Clin. Invest. **51**: 604–614.
29. **Stossel, T. P., T. D. Pollard, R. J. Mason, and M. Vaughan.** 1971. Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. J. Clin. Invest. **50**:1745–1757.
30. **Swanson, J., E. Sparks, D. Young, and G. King.** 1975. Studies on gonococcus infection. X. Pili and leukocyte association factor as mediators of interactions between gonococci and eukaryotic cells in vitro. Infect. Immun. **11**:1352–1361.
31. **Unanue, E. R., and M. J. Karnovsky.** 1973. Redistribution and fate of IgG complexes on surface of B lymphocytes: functional implications and mechanisms. Transplant. Rev. **14**:184–210.
32. **Unanue, E. R., and M. J. Karnovsky.** 1974. Ligand-induced movement of lymphocyte membrane macromolecules. V. Capping, cell movement and microtubular function in normal and lectin-treated lymphocytes. J. Exp. Med. **140**:1207–1220.
33. **Van Oss, C. J., and C. F. Gillman.** 1972. Phagocytosis as a surface phenomenon. I. Contact angles and phagocytosis on non-opsonized bacteria. RES J. Reticuloendothel. Soc. **12**:283–292.
34. **Van Oss, C. J., C. F. Gillman, and R. J. Good.** 1968. The influence of the shape of phagocytes on their adhesiveness. Immunol. Commun. **1**:627–636.
35. **Van Oss, C. J., C. F. Gillman, and A. W. Newman.** 1975. Phagocytic engulfment and cell adhesiveness as surface phenomena. Marcel Dekker, New York.
36. **White, J. G.** 1968. Fine structural alterations induced in platelets by adenosine diphosphate. Blood **31**:604–622.
37. **Wilkinson, D. C.** 1976. Recognition and response in mononuclear and granular phagocytes. Clin. Exp. Immunol. **25**:355–366.