INFECTION AND IMMUNITY, Sept. 2000, p. 5416–5419 Vol. 68, No. 9
0019-9567/00/$04.00+0

Invasion of Human Vascular Endothelial Cells by *Actinobacillus actinomycetemcomitans* via the Receptor for Platelet-Activating Factor

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Received 31 January 2000/Returned for modification 15 March 2000/Accepted 30 May 2000

Strains of the periodontal pathogen *Actinobacillus actinomycetemcomitans* are variable with respect to display of phosphorylcholine (PC)-bearing antigens. We have examined strains of *A. actinomycetemcomitans* with and without PC to assess their ability to invade endothelial cells via the receptor for platelet-activating factor (PAF). Results of antibiotic protection assays indicate that PC-bearing *A. actinomycetemcomitans* invade human vascular endothelial cells by a mechanism inhibitable by CV3988, a PAF receptor antagonist, and by PAF itself. The invasive phenotype was verified by transmission electron microscopy. A PC-deficient strain of this organism was not invasive. This property, in addition to the established ability of *A. actinomycetemcomitans* to invade epithelial cells, may provide this organism with access to the systemic circulation. The ability of PC-bearing oral bacteria to access the circulation may also explain the elevated levels of anti-PC antibody in serum found in patients with periodontitis.

Phosphorylcholine (PC) has been detected on a number of pathogenic prokaryotes, including *Streptococcus pneumoniae* and other gram-positive bacteria such as other streptococci, *Bacillus* spp., *Clostridium* spp., and other bacilli, as well as the gram-negative species *Haemophilus influenzae* (5). All of these prokaryotes contain choline within structural molecules, within either teichoic acids, lipoteichoic acids, or lipopolysaccharide (LPS). The function of such PC in pathogenesis is largely unknown for most species. However, specific examples exist that implicate PC as a virulence factor. The prototypical bacterial species containing PC is *S. pneumoniae*, which incorporates choline from culture media into PC in its teichoic acid and lipoteichoic acid (9). It has been shown that PC may mediate invasiveness of *S. pneumoniae* in the lung (1, 10, 11) and the brain (7) by permitting access of this bacterium to the receptor for platelet-activating factor (PAF) on endothelial cells. In addition, it has been suggested that PC contributes to the persistence of *H. influenzae* in the human respiratory tract (12). A genetic locus required for PC metabolism in *S. pneumoniae* has been identified which contains genes similar to a homologous locus in *H. influenzae*; mutation of some of these genes leads to decreased virulence of *S. pneumoniae* (13).

Recent studies of the oral flora and the respiratory tract flora have identified additional species which have structural molecules bearing PC (3, 4, 6); these molecules have invariably been shown to contain PC by specific reactivity with monoclonal antibodies or myeloma proteins which react only with PC. Studies performed in our laboratories (8) and an extensive survey of plaque bacteria by Gmur and coworkers (4) indicate that a significant proportion of supragingival and subgingival plaque bacteria react with TEPC-15, an immunoglobulin A myeloma protein with specificity for PC. Although the importance of PC as an antigen in oral bacteria has not been established, it has been proposed that it is a virulence factor of *S. pneumoniae* (10, 11). This idea is supported by the fact that PAF, which contains PC, is mimicked by virulent strains of *S. pneumoniae*, which access the circulatory system by binding to the PAF receptor on endothelial cells, invading these cells, and transmigrating through the endothelium into the bloodstream.

It is thought that induction of anti-PC is mainly due to exposure to *S. pneumoniae*. However, our recent findings indicate that patients with periodontal attachment loss (in all disease categories) have higher levels of anti-PC than healthy patients (8). The implication is that the oral flora is likely a source of immunogen for generation of anti-PC. These studies further show that about 40% of plaque bacteria react with TEPC-15 and thus likely contain PC. Given these observations, it is reasonable to hypothesize that some oral bacterial species behave like *S. pneumoniae*, gaining access to the circulatory system by binding to the PAF receptor on endothelial cells and inducing elevated levels of antibody to PC.

Identification of strains of *Actinobacillus actinomycetemcomitans* bearing PC. *A. actinomycetemcomitans* is a gram-negative rod that is associated particularly with early-onset periodontal diseases. This species has a wide array of virulence factors, among which is its ability to invade epithelial cells (2). We identified strains of *A. actinomycetemcomitans* bearing the PC epitope using two methods. First, the uptake of [^{3}H]choline from culture media was measured as an indicator of the relative incorporation of choline into PC in structural molecules

TABLE 1. Incorporation of [^{3}H]choline by strains of *A. actinomycetemcomitans* and *S. pneumoniae* 39937

Organism	Mean ^{3}H cpm
A. actinomycetemcomitans serotype a (SUNY Buffalo 122)	350
A. actinomycetemcomitans serotype b (VPI DB03A-42)	476
A. actinomycetemcomitans serotype c (SUNY Buffalo 360)	232
A. actinomycetemcomitans serotype b (D045D-40 [clinical isolate])	20,675
S. pneumoniae (39937)	262,289

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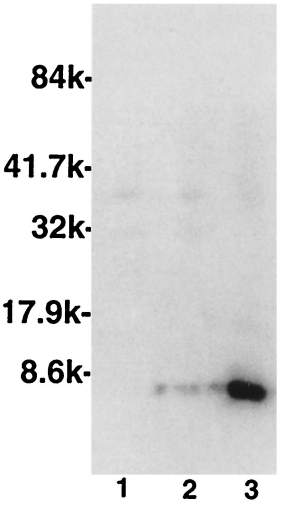

FIG. 1. Western blot analysis of TEPC-15-reactive antigens of *A. actinomycetemcomitans* DB03A-42 (lane 1), *A. actinomycetemcomitans* DR03D-03A (lane 2), and *A. actinomycetemcomitans* D045D-40 (lane 3). Molecular weights, in thousands (k), are on the left.

(8). Bacterial cultures were grown to log phase in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) containing 1.5 μCi of [^{3}H]choline chloride (New England Nuclear Life Science Products, Boston, Mass.)/ml. Cultures were washed three times with phosphate-buffered saline and resuspended in the same buffer to an optical density of 1.0 at 650 nm. Following the addition of 5 ml of scintillation cocktail (3270B; Research Products International Corp., Mount Prospect, Ill.) to 1 ml of washed bacterial suspension, the samples were subjected to scintillation counting. Data are reported as mean counts per minute for two experiments. As seen in Table 1, consistent with the data reported by Gmur et al., one of four tested strains of *A. actinomycetemcomitans* incorporated significantly greater amounts of choline than the other strains; this amount of incorporation was approximately 10% of that seen with a PC-positive strain of *S. pneumoniae*. Next, we sought to demonstrate PC-bearing antigens on these strains. We treated cultures of *A. actinomycetemcomitans* with sodium dodecyl sulfate to elute LPS from the bacterial surface and examined these antigens on immunoblots that were probed with TEPC-15 or with MOPC-315, an immunoglobulin A myeloma protein with specificity for 2,4-dinitrophenol (isotype control) as previously described (8). As shown in Fig. 1, *A. actinomycetemcomitans* D045D-40 demonstrated a pattern typical of that seen for *H. influenzae* LPS following reaction with anti-PC, where specific TEPC-15-reactive antigens are found below 8,000 kDa. Further verification of the presence of PC-bearing surface antigens was sought by performing immunofluoresence microscopy with TEPC-15 as the primary antibody; strain D045D-40 demonstrated weak positive reactivity, whereas strain DB03A-42 was unreactive. In all assays, both whole cells and eluted antigens failed to react with MOPC-315 in control experiments (data not shown). Thus, some strains of *A. actinomycetemcomitans* contain structural molecules bearing PC antigens.

Invasion of HUVEC by *A. actinomycetemcomitans*. Our previous data indicated that higher levels of anti-PC antibody in serum are present in patients with periodontitis than in unaffected controls (8). Thus, we asked how these bacteria might induce an antibody response against PC. One potential mechanism was that PC-bearing oral bacteria can gain access to the immune system, and perhaps the general circulation, via interaction with the PAF receptor on endothelial cells. To test this hypothesis, *A. actinomycetemcomitans* (at an optical density of 0.9 at 650 nm, 100 μl/well) was incubated with monolayers of human vascular endothelial cells (HUVEC) for 4 h as described by Ring and coworkers (7). Following incubation, the monolayers were washed and some cultures were treated with gentamicin (50 μg/ml, 2 h) to kill bacteria external to the HUVEC. Subsequently, the HUVEC were lysed and bacteria were plated to enumerate either total cell-associated bacteria or ingested bacteria alone. In some experiments, cultures were treated with CV3988, a synthetic competitive inhibitor of the PAF receptor, to block access of the PC-bearing bacteria to the PAF receptor. This compound was shown to have no influence on the viability of *A. actinomycetemcomitans* strains. Table 2 shows typical results of several experiments in which PC-positive *A. actinomycetemcomitans* (D045D-40) was incubated with HUVEC that had been pretreated with a PAF receptor antagonist and subsequently treated with gentamicin. The table shows the mean CFU remaining associated with the HUVEC following treatment with gentamicin to kill externalized cells. The percentage of cells internalized was calculated by dividing the CFU remaining after gentamicin treatment by the total cells associated with HUVEC prior to gentamicin treatment. The data indicate that the bacteria were internalized and that the PAF receptor antagonist inhibited internalization. In contrast, experiments with a PC-negative strain of *A. actinomycetemcomitans* (DB03A-42) indicated that this strain was not internalized (0.00%) by HUVEC. Previously, Cundell and coworkers (1) observed that invasion of HUVEC by *S. pneumoniae* was enhanced by cytokines such as tumor necrosis factor. We observed that *A. actinomycetemcomitans*

TABLE 2. Internalization of *A. actinomycetemcomitans* by HUVEC

A. actinomycetemcomitans strain	CV3988 concn (μM)	No. of CFU/ml (mean ± SD)		% of CFU	
		Bound + internalized	Internalized[a]	Compared to control[b]	Internalized[c]
D045D-40	0	3,250,000 ± 926,427	36,650 ± 5,400		1.13
	1	2,580,000 ± 473,568	28,400 ± 2,861	77	1.1
	25	1,870,000 ± 441,362	6,350 ± 1,204	17	0.34
	50	1,745,000 ± 634,849	1,655 ± 375	5	0.09
DB03A-42	0	33,500 ± 15,264	0	0	0

[a] Cultures of HUVEC containing *A. actinomycetemcomitans* were washed and treated with gentamicin. Subsequently, HUVEC were lysed and bacteria were plated to determine the number of CFU remaining.
[b] Control cultures contained no CV3988.
[c] CFU remaining following gentamicin treatment/CFU without gentamicin treatment × 100.

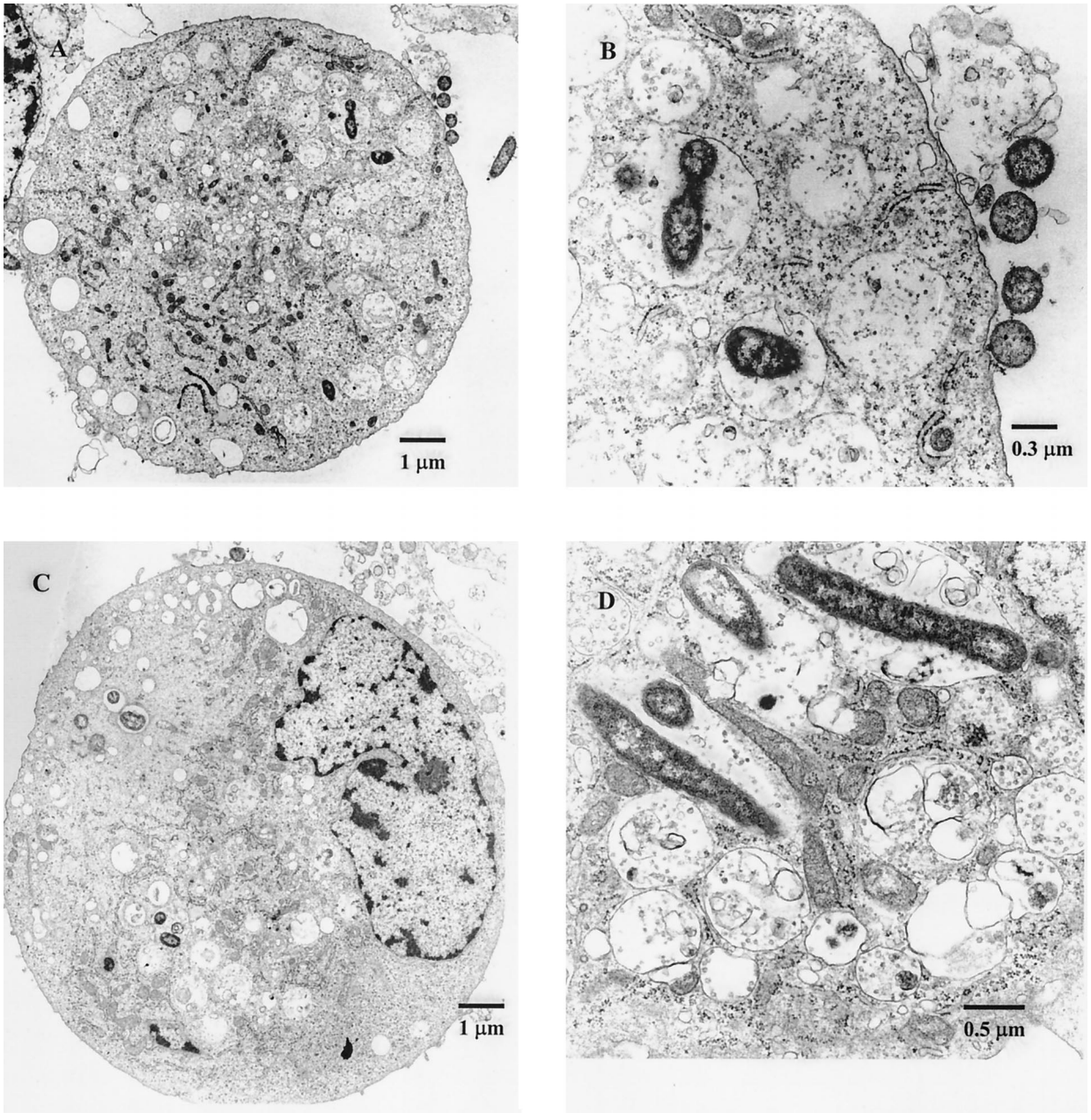

FIG. 2. Transmission electron micrographs demonstrating *A. actinomycetemcomitans* within HUVEC. (A) *A. actinomycetemcomitans* within HUVEC (magnification, ×8,100; original magnification, ×9,000); (B) Detail of panel A (magnification, ×38,900; original magnification, ×43,200); (C) HUVEC containing *A. actinomycetemcomitans* (magnification, ×8,100; original magnification, ×9,000); (D) HUVEC with internalized *A. actinomycetemcomitans* (magnification, ×38,900; original magnification, ×43,200).

readily invaded HUVEC without a great deal of modulation by previous treatment with mediators (data not shown).

We examined HUVEC from the above experiment to ensure that *A. actinomycetemcomitans* was internalized within the endothelial cells. The electron micrographs in Fig. 2 indicate that this was in fact the case, showing that the bacteria do bear the invasive phenotype. An evaluation of microscopic sections from 300 individual cells revealed that approximately 93% of microscopically intact HUVEC had at least one internalized bacterial cell following interaction with *A. actinomycetemcomitans*. Interestingly, many more bacterial cells were found within the HUVEC than was anticipated from the results of the antibiotic protection assays, indicating that, as seen with *S. pneumoniae*, a significant proportion of bacteria may be killed within endothelial cells. Alternatively, *A. actinomycetemcomitans* may multiply within the HUVEC with subsequent bacterial death, which would account for the apparent discrepancy between viable cell counts and the observed level of invasion.

The results demonstrate that *A. actinomycetemcomitans* likely invades endothelial cells via a mechanism dependent upon the engagement of the PAF receptor by bacterial PC. Extensive previous data indicate that this species invades epithelial cells via a mechanism independent of this receptor (2). In addition to the well-established ability of *A. actinomycetem-*

comitans to invade epithelial cells, we propose that PC-positive members of this species can also gain access to the circulation through intact oral tissues. This activity may be a model for invasive activity of other PC-bearing oral bacteria that otherwise gain access to underlying connective tissues, perhaps as a result of denudation and ulceration of the pocket epithelium consequent to periodontal infections.

This work was supported by grant DE10703 from the National Institute of Dental and Craniofacial Research.

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Editor: J. D. Clements