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In Vivo and In Vitro Studies on Possible Pathogenic Mechanisms of *Actinomyces viscosus*

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Actinomycotic infections are characterized by long-term inflammatory lesions containing large numbers of polymorphonuclear leukocytes (PMNs) and mononuclear cells. The pathogenic mechanisms involved in these lesions are not understood. Homogenates of *Actinomyces viscosus* (AVIS) induce an acute inflammatory response with a predominance of PMNs within 6 h after injection into the footpads of nonimmunized mice. These homogenates, when tested in vitro, contain potent chemotactic activity for human PMNs. In vitro chemotactic activity for human monocytes is weak but statistically significant ($P < 0.025$). Doses of AVIS, which alone have little chemotactic activity, cause the generation of PMN chemotactic activity in fresh, but not complement-inactivated, serum. The injection of AVIS into the footpads of immunized mice induces an acute inflammatory response followed within 48 h by a mononuclear cell infiltrate, suggesting that factors affecting monocyte accumulation are generated by the immune host in response to challenge with the bacterial antigens. These findings indicate that the pathogenicity of the *Actinomyces* may result in part from (i) their direct chemotactic effect on PMNs, (ii) their cytotaxigenic effects on serum, and (iii) their ability to stimulate host immune cells to produce and release mediators of inflammation.

The *Actinomyces* are pathogenic gram-positive bacteria which cause long-term inflammatory lesions in humans and in some animal species (4, 6, 14). A considerable amount of tissue damage and fibrosis may accompany infection. In typical lesions, there is a central zone of polymorphonuclear leukocytes (PMNs) associated with the bacteria and an outer zone containing mononuclear cells (1, 5). The pathogenesis of actinomycotic infections is not understood since these organisms are not known to elaborate exotoxins, and because their cellular components are not considered to be toxic. Tempel et al. (16) showed that culture filtrates of *Odontomyces* (*Actinomyces*) *viscosus* were chemotactic for mouse and rabbit PMNs, although the possible importance of these factors in the pathogenesis of actinomycotic infections has not been considered. To our knowledge, experiments directed toward accounting for the presence and role of mononuclear cells in the lesions have not been done. Using a mouse model system, combined with in vitro techniques utilizing human cells, we have found that *A. viscosus* homogenate (AVIS), as well as culture filtrate, has potent chemotactic activity for PMNs. In addition, AVIS generates PMN chemotactic activity when combined with fresh human serum. Although the homogenate has only weak, direct chemotactic activity for monocytes in vitro, it may induce the in vivo accumulation of mononuclear cells by triggering host immune systems, such as a delayed hypersensitivity response.

MATERIALS AND METHODS

Preparation of chemotactic substances. *A. viscosus* (strain WVU/371) was grown in brain heart infusion (BHI) medium and harvested by centrifugation after 72 h of culture. Taxonomy of *A. viscosus* was verified by gas chromatography of the volatile acids, catalase reaction, dark-field microscopy, Gram stain, and colony morphology on anaerobic blood agar plates (laboratory of Betsy Williams). The bacterial cells were washed two times in phosphate-buffered saline (PBS) and once in distilled water and were ruptured in a Braun mechanical cell homogenizer (Bronwill Scientific, Inc., Rochester, N.Y.). Cell rupture was approximately 99% complete as determined by light microscopic examination of Gram-stained samples of the bacterial suspension after homogenization for 3 to 4 min in bottles containing 3 to 4 g of microorganisms and 50 g of 0.17- to 0.18-mm glass beads (B. Braun, AG, Melsungen, Germany). Unbroken cells and glass beads were removed by centrifugation at 1,000 × *g* for 10

min. The homogenate was freeze-dried and stored at 4°C, using calcium chloride as a desiccant.

In addition, BHI medium from a 3-day culture of *A. viscosus* was filtered through a sterile 0.22-μm filter, dialyzed in Spectropor dialysis tubing (no. 132650, Spectrum Medical Industries, Inc., Los Angeles, Calif.) against distilled water, and then lyophilized. As a control, fresh BHI medium was handled in an analogous manner.

Other in vitro PMN chemotaxis controls consisted of fresh or complement-inactivated human serum made 5 or 10% (vol/vol) in Hanks balanced salt solution. For the in vitro monocyte chemotaxis experiments, a positive control of casein-saturated minimal essential medium (C-MEM) with 20% (vol/vol) fresh human serum was used. C-MEM was prepared as previously described (18). MEM alone served as a negative control in the monocyte chemotaxis assays.

In vivo assays. Two- to four-month-old female C57BL/6J mice were obtained from Jackson Laboratories, Bar Harbor, Me. To induce delayed hypersensitivity to *A. viscosus* antigens, mice were injected in the rear footpads and nuchally with 800 μg of heat-killed (60°C for 30 min) *A. viscosus* organisms suspended in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich). As a positive control for delayed hypersensitivity, some mice were immunized to *Mycobacterium tuberculosis* following the method of Phillips et al. (13).

A footpad swelling assay was used as a measure of inflammatory cell response to challenge with AVIS or purified protein derivative of tuberculin (PPD). Briefly, 50 μg of AVIS or PPD, suspended in 50 μl of PBS, was injected subcutaneously into the front footpads of nonimmunized or immunized mice. As a control, the contralateral footpad was injected with 50 μl of PBS alone. Footpad swelling was measured with a micrometer at 6 and 48 h after injection. The feet of three mice were measured at each time point, and the mean and standard deviation of changes in footpad thickness in millimeters were calculated.

Three mice from each experiment were sacrificed at 6 and 48 h postchallenge. The front feet were removed, fixed in 10% neutral buffered formalin, and embedded in paraffin, and histological sections were prepared. The extent of inflammatory cell infiltrate and the predominant inflammatory cells present at each time period were determined microscopically.

In vitro assays. Human PMNs were prepared from heparinized (10 U/ml) normal human peripheral blood by Plasmagel (Roger Bellon Laboratories, Neuilly, France) sedimentation of erythrocytes as previously described (17, 18). The leukocytes were washed with Hanks balanced salt solution and adjusted to a concentration of 7×10^6 PMNs per ml. Approximately 60 to 65% of the cells in this preparation were PMNs. Monocyte-enriched preparations were obtained by Ficoll-Hypaque centrifugation (3), washed with MEM, and adjusted to a cell concentration of 2×10^7 cells per ml. This preparation contained from 10 to 39% monocytes.

PMN chemotactic assays were performed using a modification of the Boyden chamber technique (2) as previously described (17). Briefly, a 5-μm membrane (Millipore Corp., Bedford, Mass.) was used to separate the upper and lower compartments of the chamber. Chambers containing PMNs and substances to be tested for chemotactic activity were incubated at 37°C for 2 h. The membranes were then removed, formaldehyde-fixed, and stained. The number of PMNs that had migrated to the distal side of the membrane was determined. Assays for monocyte chemotaxis were performed in a similar fashion but with mononuclear indicator cells in MEM and a 3-h incubation. Chemotaxis was determined visually at a microscopic magnification of ×400 for the PMN assays and ×1,000 for the monocyte assays. Five randomly selected microscopic fields from each of duplicate membranes (total of 10 fields) were counted in each assay, and the mean (± standard error of the mean) number of cells per field was calculated.

RESULTS

In vivo inflammatory response. Injection of AVIS into the footpads of normal, nonimmunized mice induced an acute inflammatory response which was evident by 6 h (Fig. 1A). This response was characterized by a dense accumulation of PMNs (Fig. 1B) and swelling of the footpad (Table 1). By 48 h, footpad swelling had diminished in the nonimmunized mice and only a few PMNs remained; a mononuclear cell infiltrate did not occur.

Injection of AVIS into the footpads of mice immunized to *A. viscosus* also caused footpad swelling (Table 1) and a predominantly PMN infiltrate at the challenge site within 6 h. However, in contrast to the nonimmunized mice, footpad swelling remained basically unchanged at 48 h, and an inflammatory cell infiltrate made up primarily of mononuclear cells appeared (Fig. 2A and B).

As expected, mice immunized with *M. tuberculosis* and challenged with PPD showed little evidence of an inflammatory response at 6 h. However, by 48 h postchallenge profound footpad swelling had occurred, accompanied by a dense mononuclear cell infiltrate (Table 1).

In vitro chemotactic responses. *A. viscosus* culture filtrate and AVIS were assayed for the presence of substances chemotactic for human PMNs. As shown in Fig. 3, undialyzed culture filtrate of *A. viscosus* was chemotactic for human PMNs. However, this chemotactic activity was lost upon dialysis. Since the dialysis membrane (Spectropor) used in this study retained molecules of greater than 6,000 to 8,000 daltons, the chemotactant released into the growth medium by *A. viscosus* would appear to be a small molecule.

The homogenate of *A. viscosus* organisms also exhibited potent chemotactic activity for

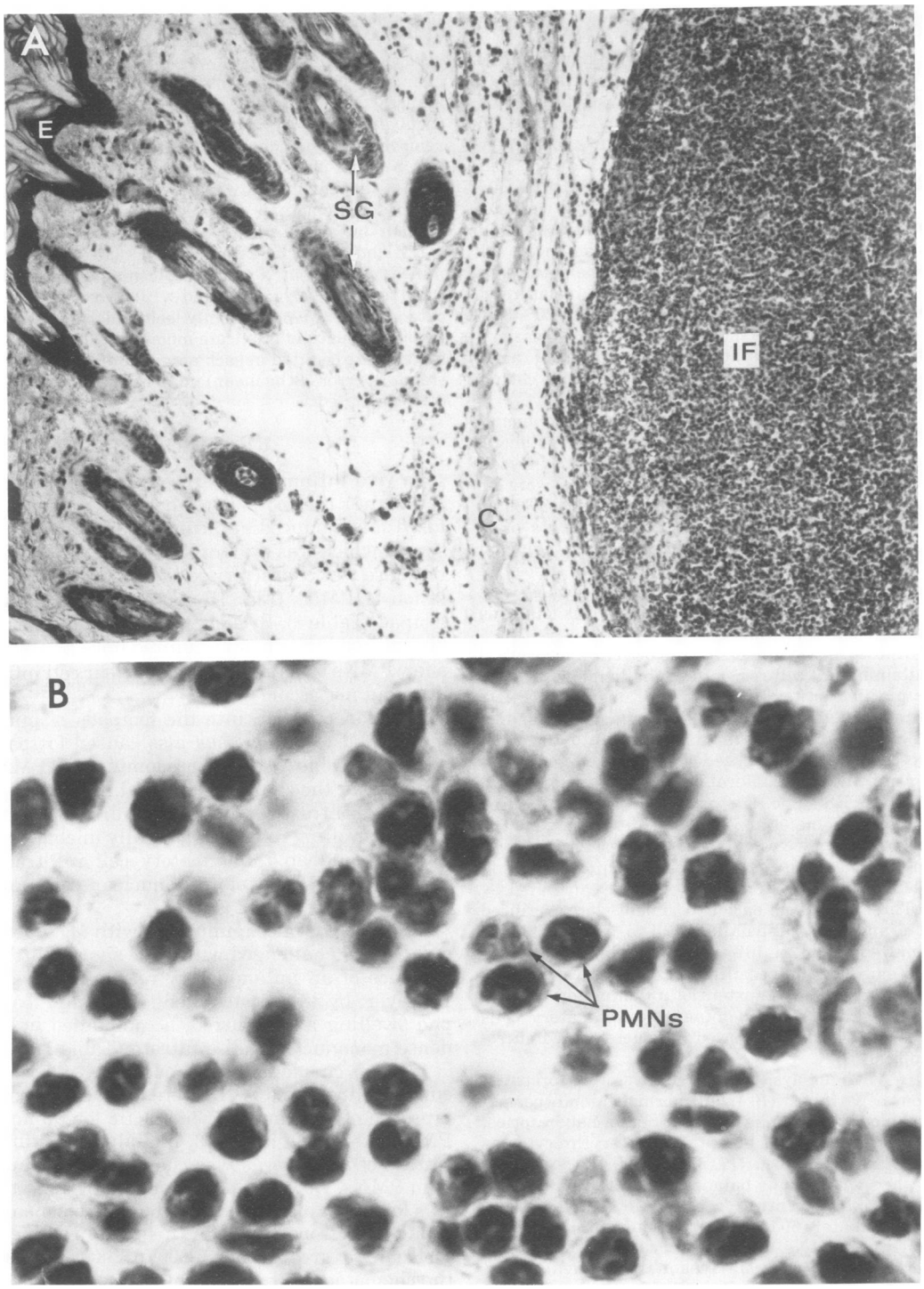

FIG. 1. *(A) An acute inflammatory cell infiltrate (IF) occurs within 6 h after the intradermal injection of 50 μg of AVIS into the footpads of immunized or nonimmunized mice. Prominent bundles of collagen (C) and sweat glands (SG) are seen lying between the epidermis (E) and infiltrate (hematoxylin-eosin, ×125). (B) The inflammatory cells responding to the AVIS challenge at 6 h are primarily polymorphonuclear leukocytes (PMNs) in both immunized and nonimmunized mice (hematoxylin-eosin, ×1,250).*

TABLE 1. *Comparisons of footpad swelling after challenge of immunized and nonimmunized mice with AVIS*

Immunization	Challenge	Increase in footpad thickness[a]		Predominant cell type	
		6 h	48 h	6 h	48 h
None	AVIS	0.33 (0.08)	0.10 (0.09)	PMN	PMN(few)
Actinomyces viscosus	AVIS	0.20 (0.09)	0.23 (0.06)	PMN	Mononuclear
Mycobacterium tuberculosis	PPD	−0.10 (0.10)	0.62 (0.03)	ND[b]	Mononuclear

[a] Net millimeters of swelling (thickness of foot receiving antigen in PBS minus thickness of foot receiving PBS alone) expressed as mean (± standard deviation) of values obtained from triplicate samples.
[b] ND, Experiment not done.

human PMNs, which was directly proportional to the dose tested over a concentration range of 0.0625 to 1.0 mg/ml (Fig. 4). This chemotactic activity was not dependent upon the presence of fresh serum.

AVIS has the capacity to generate PMN chemotactic activity when incubated with fresh human serum (Table 2). Although all fresh sera tested had some PMN chemotactic activity alone, activity was greatly increased by preincubating the sera with AVIS (0.0625 mg/ml). No activity was generated when serum complement was first inactivated by heating the sera at 56°C for 30 min (Table 2). This suggests that AVIS may generate complement-derived chemotactic factors. Differences in the magnitude of chemotactic activity generated in the four sera tested were noted (Table 2) and may have been due to differences in concentrations of complement components, although this was not tested.

Mononuclear cells, including macrophages, are a significant component of actinomycotic lesions, and bacterial chemotactic factors for monocytes have been described (20, 24). Therefore, AVIS was assayed for direct monocyte chemotactic activity. In two experiments this activity was slightly greater than that of negative controls (Table 3). These differences were evaluated for statistical significance by the *t*-test for independent means and were found to have P values of between 0.01 and 0.025.

DISCUSSION

The *Actinomyces* are associated with long-term inflammatory lesions, characterized by an accumulation of PMNs, macrophages, and plasma cells. In addition, certain *Actinomyces* induce periodontal destruction in conventional and gnotobiotic rodents (7, 9, 10, 15) and form a major component of some human dental plaques (25). Actinomycotic infections commonly lead to extensive soft tissue damage and scar formation. Although PMNs and macrophages contribute in a major way to defense of the host against bacterial infection, it is now clear that they may also contribute to tissue damage by release of their hydrolytic enzymes (8, 11, 12, 19, 23). Thus, an understanding of the mechanisms by which PMNs and mononuclear cells are attracted to the site of inflammatory lesions is essential to the understanding of both the pathogenesis of actinomycotic lesions and host resistance to *Actinomyces* infections.

Our data show that *A. viscosus* contains and releases a factor(s) chemotactic for human polymorphonuclear leukocytes. The factor(s) may be responsible in major part for the PMN component of actinomycotic lesions and for the rapid accumulation of PMNs at the site of injection of *A. viscosus* substances into mice, as was observed in our study and in a previous study (16).

The chemotactic substance(s) present in culture filtrates of *A. viscosus* appears to have a molecular weight of less than 6,000 to 8,000 since activity was lost upon dialysis using membranes that retain molecules of equal or greater mass. This is consistent with the finding by Tempel et al. (16) that the chemotactant in *A. viscosus* culture filtrates had a molecular weight of less than 12,500, as determined by sucrose density-gradient ultracentrifugation.

AVIS is not only chemotactic by itself, but also generates chemotactic activity when incubated with fresh human serum. Because this cytotoxigenic reaction is abolished by first heat inactivating the serum, it seems likely that complement is involved. The leukotactic properties of C3 and C5 fragments and of the trimolecular complex $C\overline{567}$ are well established (22). Liberation of complement-derived chemotactic factors in these experiments theoretically may have occurred by any one of at least three different pathways: (i) the activation of complement by AVIS via the alternate pathway (U.R. Nilsson, C.C. Tsai, W.P. McArthur, and N.S. Taichman, J. Dent. Res. 54:122, 1975); (ii) the interaction of AVIS and complement-fixing, anti-bacterial antibodies which may have been present in the sera tested; or (iii) direct cleavage of complement by proteinases, which are found in some bacteria (21).

The appearance of large numbers of mononuclear cells in actinomycotic lesions probably cannot be accounted for solely by direct chemo-

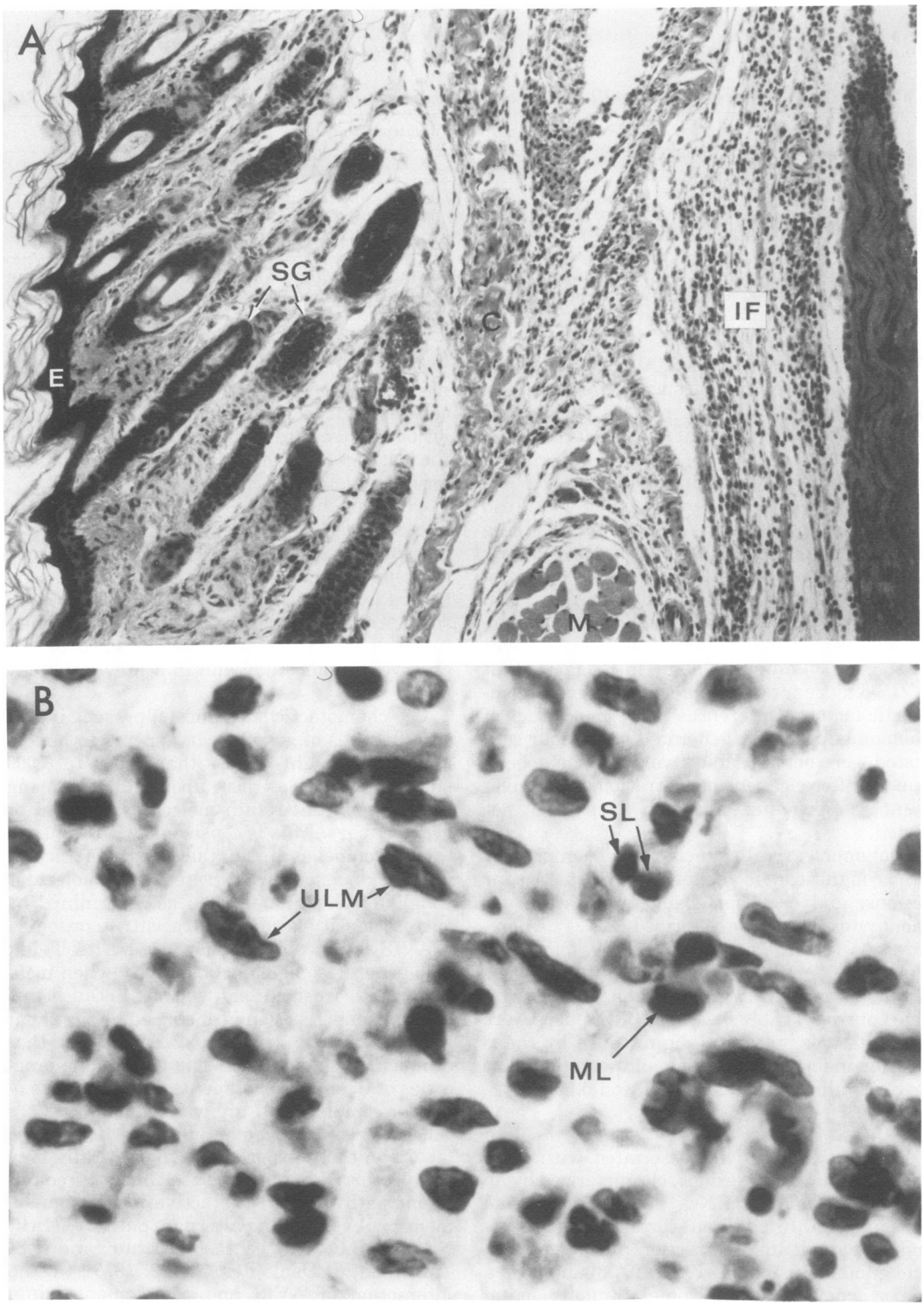

FIG. 2. *(A) Mice immunized to A. viscosus respond with an inflammatory cell infiltrate (IF) characteristic of a delayed hypersentivity reaction 48 h after the injection of 50 μg of AVIS into the footpad. Muscle (M) and collagen bundles (C), as well as numerous sweat glands (SG), lie between the epidermis (E) and the infiltrate (hematoxylin-eosin, ×125). (B) Mononuclear cells are the predominant cell type responding to the AVIS challenge at 48 h in immunized mice. Cells consistent in appearance with small lymphocytes (SL) and medium lymphocytes (ML), as well as numerous unidentified large mononuclear cells (ULM), are seen (hematoxylin-eosin, ×1,250).*

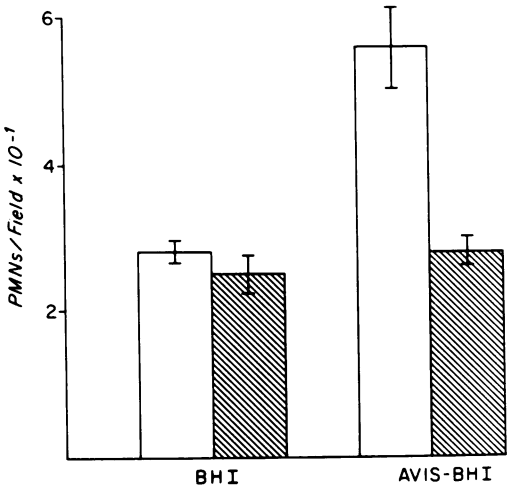

FIG. 3. *Release of PMN chemotactic activity into the culture medium by A. viscosus. The PMN chemotactic activity of dialyzed and undialyzed fresh brain heart infusion medium (BHI) (1 mg/ml in MEM) and the same concentration of dialyzed and undialyzed medium from a 3-day culture of A. viscosus (AVIS-BHI) are shown. Values are expressed as the mean (± standard error of mean) number of human PMNs per microscopic field × 10^{-1}. Symbols: □, undialyzed, ▨, dialyzed.*

tactic effects of the bacteria upon monocytes. Monocyte chemotactic activity of AVIS in vitro, although statistically significant, was slight when compared to the positive control. Furthermore, on the basis of our in vivo experiments, generation of monocyte chemotactic factors by activation of complement components or by release of basic lysosomal peptides from neutrophils (22) seems unlikely since AVIS challenge of nonimmunized mice did not elicit a mononuclear cell infiltrate at either 6 or 48 h. Rather, our data support the contention that the mononuclear infiltrate may result from a delayed-type hypersensitivity reaction, since only immunized mice elicited a monocyte infiltrate 48 h after challenge with *A. viscosus* antigens. Nevertheless, *Actinomyces* substances may have an important direct effect upon monocytes once they arrive at the site of an infection, since macrophages exposed to AVIS in vitro are stimulated to synthesize and secrete potentially tissue-damaging hydrolytic enzymes (11).

Plasma cells, the remaining predominant constituent of long-term actinomycotic lesions, may arise as a result of stimulation of B-lymphocytes by specific *A. viscosus* antigens. Alternatively, AVIS has recently been found to contain a potent B-cell mitogen (D. Engel, J. Clagett, B. Williams, and R. Page, Fed. Proc. 35:823, 1976) which has the capacity to drive resting B-cells to the plasma cell stage (J. Clagett, D. Engel, and E. Chi, Fed. Proc. 35:592, 1976). The role of plasma cells in the pathogenesis of the lesion is yet unclear.

The observations described suggest that the *Actinomyces* may induce disease by activating various host-defense mechanisms. These mechanisms, besides limiting the bacterial infection, may contribute to the observed tissue damage.

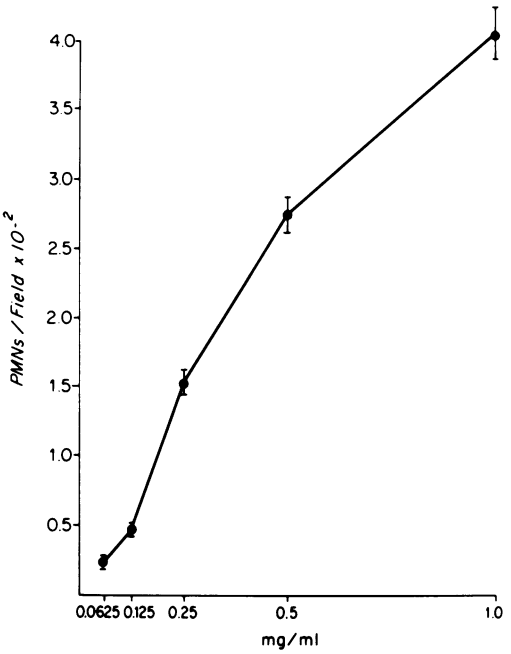

FIG. 4. *In vitro dose-response curve for direct PMN chemotactic activity of various concentrations of AVIS suspended in MEM (mg/ml). Values are reported as the mean (± standard error of mean) number of human PMNs per microscopic field × 10^{-2}.*

TABLE 2. *Generation of PMN chemotactic activity in vitro by interaction of AVIS with fresh, normal human serum*

Serum donor	Chemotactic activity[a]				
	Chemotactant[b]				
	AVIS + FS	AVIS + CIS	AVIS	FS	CIS
1	288 (16)	ND[c]	14 (3)	87 (9)	ND
2	116 (11)	ND	14 (3)	72 (8)	ND
3	181 (12)	22 (3)	3 (1)	17 (2)	6 (1)
4	47 (3)	8 (1)	3 (1)	18 (3)	3 (1)

[a] Expressed as the mean (± standard error of mean) number of human PMNs per microscopic field (×400).

[b] Fresh serum (FS) or complement-inactivated serum (CIS) (56°C for 30 min) was incubated at 37°C for 1 h with AVIS (0.0625 mg/ml) before assaying for chemotactic activity. Serum concentrations were 10% for donors 1 and 2 and 5% for donors 3 and 4 ([vol/vol] in Hanks balanced salt solution). The same concentrations of AVIS, FS, and CIS alone were assayed separately as controls.

[c] ND, Experiment not done.

TABLE 3. *In vitro monocyte chemotactic activity of AVIS*

Expt	Chemotactic activity[a]		
	Chemotactant		
	Positive control[b]	AVIS[c]	Negative control[d]
1	11.8 (1.3)	1.7 (0.5)	0.5 (0.2)
2	13.6 (1.3)	2.0 (0.4)	0.7 (0.4)

[a] Expressed as the mean (± standard error of mean) number of human monocytes per microscopic field (×1000).

[b] Casein-saturated MEM containing 20% (vol/vol) fresh human serum.

[c] AVIS at a concentration of 1 mg/ml of MEM.

[d] MEM alone.

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