INFECTION AND IMMUNITY, Nov. 1984, p. 367–371
0019-9567/84/110367-05$02.00/0

Vol. 46, No. 2

Enhancement of Experimental Actinomycosis in Mice by *Eikenella corrodens*

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Received 9 May 1984/Accepted 3 August 1984

The infectivity of *Actinomyces israelii* in a susceptible-weanling-mouse model was increased by the presence of *Eikenella corrodens* in the inoculum. A minimal infecting dose of 1.7×10^7 CFU of *A. israelii* was required to establish chronic lesions after an intraperitoneal injection. When *E. corrodens* (3.8×10^7 CFU) was included in the inoculum, chronic lesions were established with a dose of 8.5×10^4 CFU of *A. israelii*. *E. corrodens* alone did not produce persistent lesions. Viable *E. corrodens* could be recovered from chronic mixed actinomycotic lesions in numbers that often equaled or exceeded the populations of *A. israelii* in the lesions. The duration of acute actinomycotic infections caused by *A. viscosus* was temporarily extended by the presence of *E. corrodens*. The cellular inflammatory response and overall morphology of mixed experimental lesions containing *A. israelii* and *E. corrodens* did not appear to be significantly different from those of pure-culture lesions containing *A. israelii* alone. *E. corrodens* cells could not be readily discerned in stained histological sections of mixed experimental lesions.

Gram-negative bacteria such as *Eikenella corrodens*, *Actinobacillus actinomycetemcomitans*, *Bacteroides* spp., and *Fusobacterium* have been found in association with *Actinomyces* spp. in human actinomycosis (5, 6, 9–11, 14, 15). Little is known about the role of these associated pathogens in actinomycosis; however, it has been speculated, on the basis of clinical observations, that the infection results from a synergistic action between the *Actinomyces* spp. and the accompanying gram-negative organisms (10).

Actinomycotic infections that resemble natural actinomycosis can be produced experimentally in a susceptible-weanling-mouse model with pure cultures of *Actinomyces israelii*, *A. naeslundii*, and *A. viscosus* (2, 4, 7, 8). Mixed actinomycotic infections have been produced in this model with *A. israelii* in combination with *Eikenella corrodens* or *A. actinomycetemcomitans* (12). The gram-negative bacteria persisted in the chronic experimental lesions in association with *A. israelii* but were unable to survive alone in pure-culture lesions in the same animal model. This lends partial support to the idea of a mutually beneficial interaction between *Actinomyces* spp. and other microbial components in a mixed actinomycotic infection.

The purpose of the present work was to examine additional aspects of microbial interactions in mixed actinomycotic infections and, specifically, to assess the potential contributions of an associated gram-negative pathogen to the infection.

MATERIALS AND METHODS

Bacterial strains. *A. viscosus* M100 and *A. israelii* 12102 were revived from the lyophilized state and cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Both strains were incubated anaerobically in Square-Pak Flasks (American Sterilizer Co., Erie, Pa.) (3) at 37°C. The length of incubation was 2 days for *A. viscosus* and 4 days for *A. israelii*. *E. corrodens* 1073 was grown in Trypticase soy broth plus sodium succinate (500 mg/liter), hemin (5 mg/liter), and yeast extract (5 g/liter). Incubation was carried out in Brewer anaerobic jars under an atmosphere of 80% N_2–10% H_2–10% CO_2 for 2 days at 37°C. Stock cultures of these three strains were maintained as frozen broth cultures with glycerol added to a final concentration of 50%.

Cells to be used for animal inoculation were harvested from the broth by centrifugation, washed twice with phosphate-buffered saline, and resuspended in phosphate-buffered saline to an optical density estimated to provide ca. 10^8 CFU/ml. Viable cell populations in the various inoculum preparations were determined by culturing on Trypticase soy agar containing 5% sheep blood.

Animals. Experimental actinomycotic infections were established in male CD-1 Swiss mice (Charles River Breeding Laboratories, Wilmington, Mass.). Weanling animals ca. 21 days old and weighing not over 10 to 15 g were used. They were housed in polypropylene mouse cages containing pine sawdust bedding. Purina Mouse Chow (Ralston Purina Co., St. Louis, Mo.) and distilled water were provided ad libitum.

Infectivity of *A. israelii*. Graded inoculum doses of *A. israelii* were tested for their ability to induce experimental lesions alone and in the presence of a uniform inoculum dose of *E. corrodens*. Serial 10-fold dilutions of *A. israelii* 12102 which spanned the range of 3.4×10^8 to 3.4×10^4 CFU/ml were prepared in saline. Groups of 30 weanling mice were injected intraperitoneally with the appropriate cell suspension at 0.5 ml per animal.

Samples from the same *A. israelii* dilutions were combined with equal volumes of a standard cell suspension of *E. corrodens* 1073 (7.5×10^7 CFU/ml). The mixed inocula were tested in the same way as the *A. israelii* pure-culture inocula relative to the volume of the inoculum, the route of injection, and the number of animals. *E. corrodens*, by itself, was tested in an additional group of animals at a dose of 7.5×10^7 CFU in a 0.5-ml cell suspension.

Subgroups of 10 animals each from each of the experimental groups were sacrificed in a CO_2 chamber at 2, 6, and 12 weeks. Internal organs were examined for the presence and location of actinomycotic abscesses. Sham-inoculated controls were not included in the present experiments, as this procedure has consistently yielded negative results in the past (2, 12).

Populations of viable organisms in lesions. Randomly selected lesions were removed aseptically at autopsy and

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TABLE 1. Comparative infectivity of *A. israelii* 12102 alone and in the presence of *E. corrodens* 1073

Infecting organism	(CFU of *A. israelii*) Inoculum	Wk 2		Wk 6		Wk 12	
		Proportion of animals infected	Avg no. of lesions per animal	Proportion of animals infected	Avg no. of lesions per animal	Proportion of animals infected	Avg no. of lesions per animal
A. israelii	1.7×10^8	8/8	18.1	6/7	18.1	7/8	7.6
	1.7×10^7	10/10	5.1	7/8	3.4	2/9	1.0
	1.7×10^6	1/10	1.0	0/9	0	0/9	0
	1.7×10^5	0/9	0	0/9	0	0/8	0
E. corrodens	7.5×10^7	3/7	1.7	1/6	1.0	0/6	0
A. israelii + *E. corrodens*	8.5×10^{7a}	6/6	5.3	5/6	3.0	5/7	2.6
	8.5×10^{6a}	9/9	2.3	10/10	2.4	6/10	2.1
	8.5×10^{5a}	8/9	3.6	8/10	2.5	8/10	1.1
	8.5×10^{4a}	7/9	2.0	7/9	1.3	5/10	1.8

[a] Each dilution also contained 3.8×10^7 CFU of *E. corrodens*.

homogenized in saline with Ten Broeck tissue grinders. Appropriate dilutions were cultured in duplicate on Trypticase soy-blood agar. The culture plates were incubated at 37°C for 7 days under an atmosphere of 80% N_2–10% H_2–10% CO_2 in Brewer anaerobic jars. Estimates of viable bacterial populations in the lesions were obtained from mean colony counts on suitable dilution plates.

***E. corrodens* influence in acute lesions.** *A viscosus* is known to produce only short-term acute lesions that do not progress to the chronic stage in the mouse model (2). *A. viscosus* M100 was tested alone and in combination with *E. corrodens* 1073 to see whether the presence of an associated gram-negative pathogen would enhance the severity or prolong the existence of acute lesions caused by this less pathogenic *Actinomyces* species. Weanling mice in an experimental group of 30 animals were injected with 0.5 ml of an inoculum suspension of *A. viscosus* M100 containing 1.2×10^8 CFU. Mice in a second experimental group were injected with 0.5 ml of an inoculum suspension of *E. corrodens* 1073 containing 1.1×10^8 CFU. Mice in a third experimental group received 0.5 ml of a mixed inoculum of the two organisms prepared by combining equal volumes of each suspension. Subgroups of 10 animals each were sacrificed, autopsied, and examined for the presence and numbers of lesions at 1, 2, and 4 weeks.

Histology. Immediately after sacrifice, representative lesions from animals in each experimental group were removed and fixed in 5% buffered Formol-saline for 24 h. The specimens were washed, dehydrated in graded alcohols, and embedded in paraffin. Paraffin blocks containing the lesions were sectioned (6-μm sections), and serial sections were stained with hematoxylin and eosin and the Gram-Weigert and Brown and Brenn stains for bacteria in tissue sections. Additional histochemical tests that have been used for detecting gram-negative bacteria in tissue sections were also used. These included Krajian's modification (13) of the Ziehl-Neelsen technique and the Gram-Weigert stain counterstained with carbol-fuchsin. All sections were evaluated with a light microscope.

Central granules of mixed lesions were stained and examined directly for the presence of *E. corrodens*. Abscesses obtained at 6 weeks were cut open, and the contents were collected in tubes of saline. The mixtures were shaken, and the granules were harvested after settling. After repeated washings in saline to remove polymorphonuclear leukocytes, the remaining granules were fixed in Formol-saline. For microscopic examination, small samples containing granules were crushed between two glass slides. The smear thus obtained was air dried, heat fixed, and Gram stained, with carbol-fuchsin as a counterstain (6).

RESULTS

Infectivity of *A. israelii*. The infectivity of *A. israelii* was increased significantly by the presence of *E. corrodens* in the inoculum. A minimum dose of 1.7×10^7 CFU was required for *A. israelii* 12102, when tested by itself, to establish typical actinomycotic lesions in this experiment. The same strain of *A. israelii*, when tested in the presence of *E. corrodens*, was infective at a dose of 8.5×10^4 CFU, the highest inoculum dilution tested. *E. corrodens*, when tested by itself, did not induce persistent lesions. The results of this experiment are shown in Table 1. The presence of *E. corrodens* in the inoculum did not appear to cause any increase in the number or size of lesions or to result in any unusual spread of lesions to extraabdominal sites.

Populations of viable organisms in lesions. All lesions from animals infected with *A. israelii* alone contained viable organisms, whereas none of the lesions caused by *E. corrodens* alone harbored viable organisms. The comparatively few lesions in animals infected with *E. corrodens* were

TABLE 2. Comparative populations of *A. israelii* 12102 and *E. corrodens* 1073 in experimental actinomycotic lesions

Lesion type	Infecting agent	Mean CFU × 10^6 (±SD) recovered per lesion at:		
		2 wk	6 wk	12 wk
Pure culture[a]	*A. israelii*	3.7 (±3.7)	7.1 (±8.6)	4.5 (±7.6)
Mixed[b]	*A. israelii*	3.1 (±4.1)	3.4 (±7.0)	12.2 (±23.1)
	E. corrodens	12.7 (±19.9)	4.1 (±10.5)	2.3 (±3.3)

[a] Mean values for *A. israelii* pure-culture lesions were based on two to three lesions from the two highest inoculum doses tested.
[b] Mean values for *A. israelii-E. corrodens* mixed lesions were based on three to four lesions from each of the inoculum doses tested (see Table 1).

TABLE 3. Influence of *E. corrodens* 1073 on acute actinomycotic lesions caused by *A. viscosus* M100

Infecting organism (CFU)	Wk 1		Wk 2		Wk 4	
	Proportion of animals infected	Avg no. of lesions per animal	Proportion of animals infected	Avg no. of lesions per animal	Proportion of animals infected	Avg no. of lesions per animal
A. viscosus (1.2×10^8)	8/10[a]	2.9	5/10[a]	2.4	7/10	2.0
E. corrodens (1.1×10^9)	7.10[a]	3.4	6.10[a]	1.5	2/10	1.0
A. viscosus (6×10^7) + *E. corrodens* (5.5×10^8)	10/10[a]	6.0	10/10[a]	3.5	9/10[a]	2.1

[a] Viable organisms were recovered from lesions.

considered to be microbiologically inactive at 2 weeks, even though they were not yet physically resolved. All mixed lesions induced by the combination of *A. israelii* and *E. corrodens* contained viable organisms of both species. The relative populations of viable organisms recovered from pure-culture *A. israelii* lesions and mixed *A. israelii-E. corrodens* lesions are shown in Table 2. These results reflect the wide variations in bacterial numbers and indicate that the mixed actinomycotic lesions harbored significant populations of *E. corrodens* that often equaled or exceeded the numbers of *A. israelii*.

***E. corrodens* influence in acute lesions.** Acute infections caused by *A. viscosus* were enhanced by the presence of *E. corrodens* in the inoculum. This was manifested by a higher percentage of animals with lesions, a higher average number of lesions per animal, particularly at 1 and 2 weeks, and an extended persistence of microbiologically active lesions. The size of the lesions was not affected. More than 50% of the animals injected with *A. viscosus* or *E. corrodens* alone developed lesions from which viable organisms were recovered at 1 and 2 weeks but not at 4 weeks. The combined inoculum of *A. viscosus* and *E. corrodens* induced lesions in 100% of the animals at 1 and 2 weeks, and 90% of the animals still had active lesions at 4 weeks. Viable organisms of both species were recovered from the mixed lesions at all three sampling periods, although the numbers declined with time, and viable numbers were very sparse at 4 weeks. The results of this experiment are shown in Table 3.

Histology. Experimental actinomycotic lesions caused by mixtures of *A. israelii* and *E. corrodens* were generally similar in their histological appearance to pure-culture lesions caused by *A. israelii* alone. Each lesion contained one or more central bacterial granules containing long, intermeshed bacterial filaments. The granules were surrounded

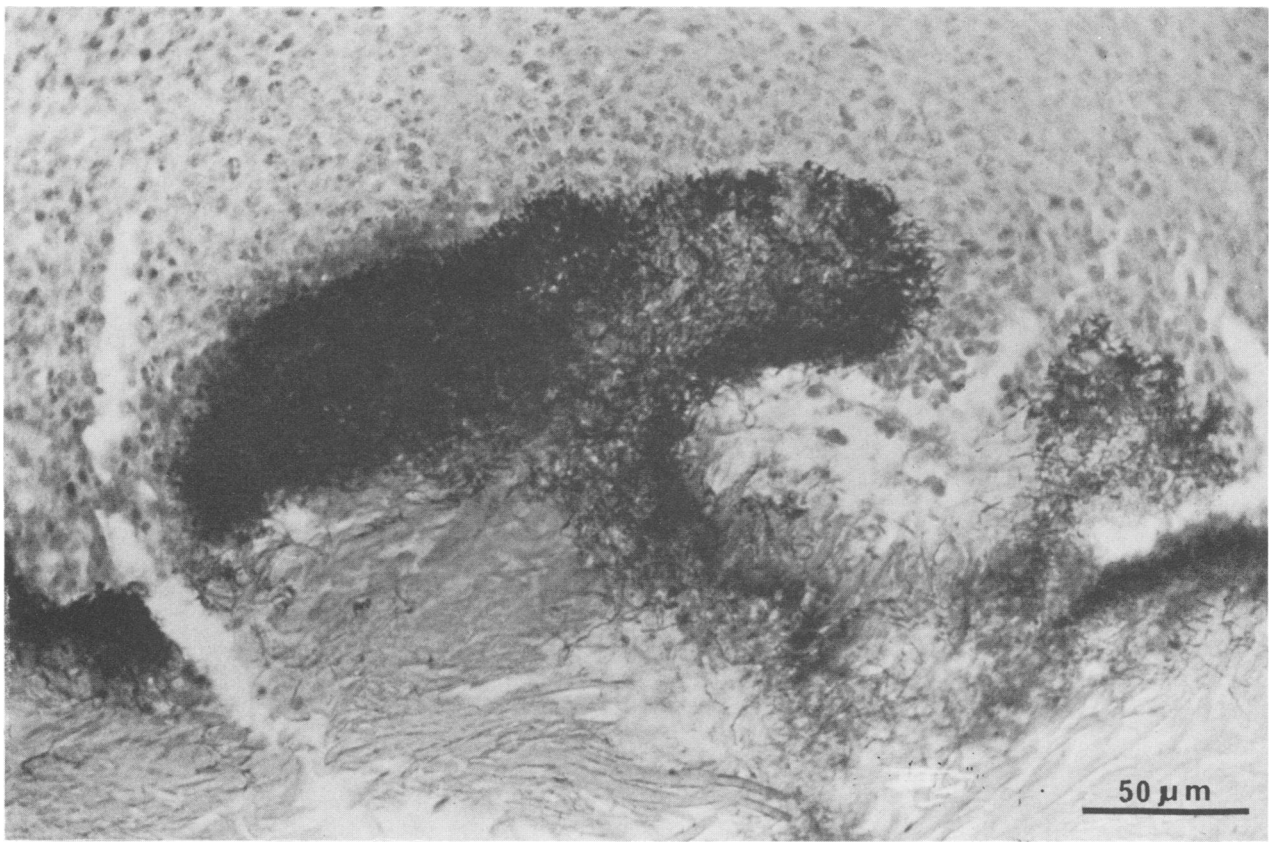

FIG. 1. Photomicrograph of a section through a mixed lesion from an animal infected with *A. israelii* and *E. corrodens* 6 weeks before sacrifice. Note the dense concentration of gram-positive, filamentous actinomycetes, particularly at the interface between the periphery of the bacterial granule and the surrounding polymorphonuclear leukocytes. Gram-Weigert stain; ×440.

by a wide zone of polymorphonuclear leukocytes (20 to 30 cells thick). Surrounding this was a zone of loosely packed, mixed inflammatory cells, including lymphocytes, macrophages, and eosinophils. The outermost zone contained fibroblasts and thin, scattered collagen fibers. The histological appearance of experimental actinomycotic lesions in the mouse model has been well described previously (1, 4). The general morphology of the lesions and the cellular character of the inflammatory response did not appear to be altered by the presence of *E. corrodens*. Gram staining demonstrated the filamentous nature of *A. israelii* in the central granule but did not reveal *E. corrodens* (Fig. 1). Sections of lesions stained by the Krajian method (13) demonstrated aggregates of positive-staining material within the central granule. However, it was not possible to recognize with certainty individual cells of *E. corrodens* within the stained aggregates (Fig. 2). Similar carbol-fuchsin-stained aggregates could be seen in abscesses caused by *E. corrodens* at 2 weeks, but individual cells were not recognizable. Smears of granules that had been harvested from lesions and crushed on glass slides showed a background of small, rod-shaped cells stained with carbol-fuchsin together with the larger, branching filaments of *A. israelii*.

DISCUSSION

The availability of a reliable animal model of actinomycosis has made it possible to investigate certain aspects of the microbiology of this disease at the experimental level. Previous studies with this model have revealed fundamental differences in pathogenicity among the various species of *Actinomyces*. Rough strains of *A. israelii* induced typical, slowly progressing, chronic actinomycotic infections, whereas *A. viscosus*, *A. naeslundii*, and smooth *A. israelii* strains induced acute lesions that were eventually resolved by the host (2). These experimental results support previous clinical observations that *A. israelii* is the principal etiological agent of human actinomycosis (16).

Recent studies on mixed actinomycotic infections in the mouse model have provided experimental evidence of mutualism between the different types of pathogens in the lesion. The gram-negative organisms *E. corrodens* and *A. actinomycetemcomitans* were found to persist in chronic actinomycotic lesions in association with *A. israelii* even though they were unable to survive in acute lesions by themselves (12). The mechanism of this apparent protective effect is not known, but histological evidence indicates that polymorphonuclear leukocytes do not penetrate and invade the developing central bacterial granule of *A. israelii* lesions (1). Thus, any organisms associated with *A. israelii* inside the granule would be isolated from direct contact with phagocytic cells.

The present investigation suggests that actinomycosis also involves the converse aspect of bacterial mutualism in which the existence of the *Actinomyces* sp. is favorably influenced by its association with the gram-negative organism. In this case, the ability of *Actinomyces* cells to survive and become established in extraoral sites would be greatly enhanced by

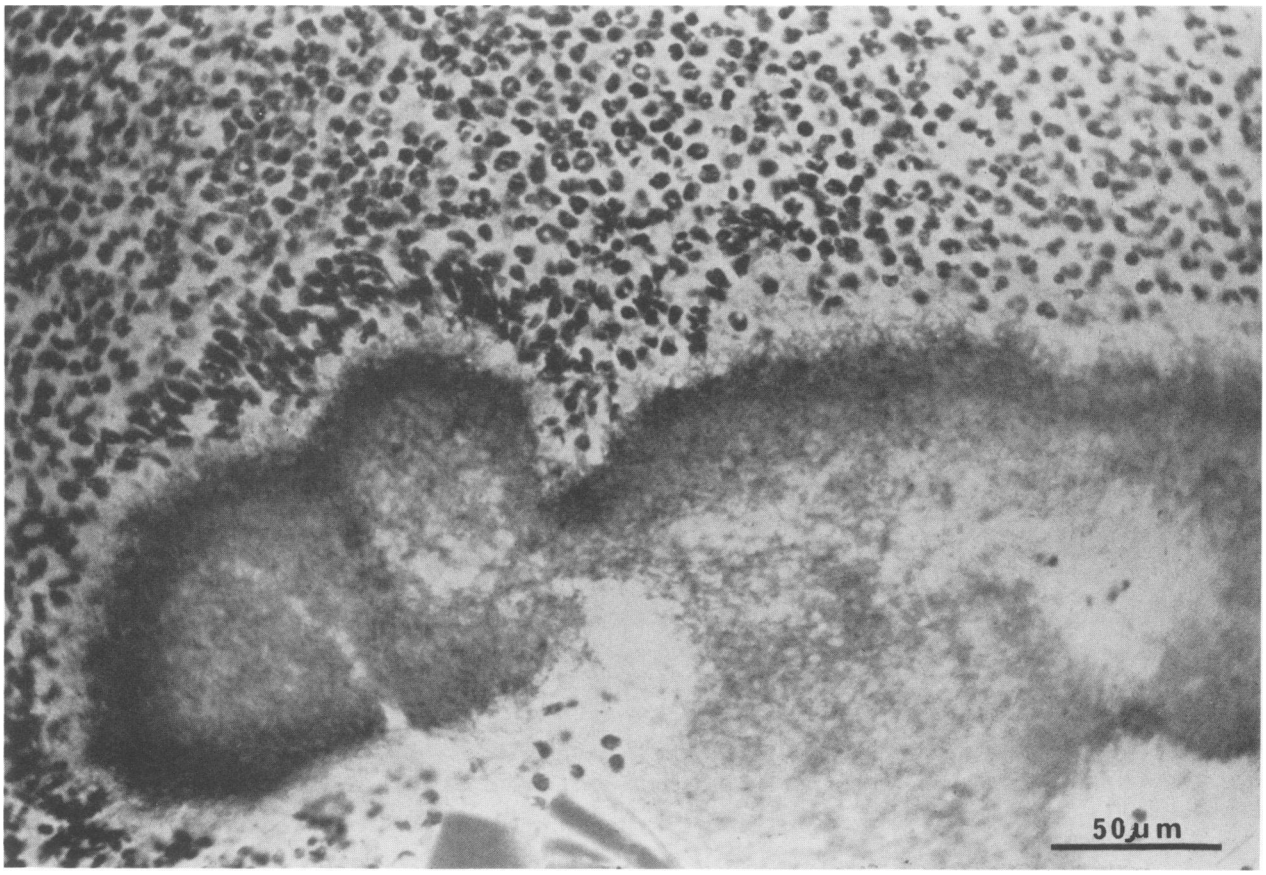

FIG. 2. Photomicrograph of a section through a mixed lesion from an animal infected with *A. israelii* and *E. corrodens* 6 weeks before sacrifice. In this section, stained for gram-negative organisms, an actinomycotic fringe surrounds the central bacterial granule. Within the granule are numerous carbol-fuchsin-stained aggregates, although individual gram-negative organisms cannot be seen. Krajian stain; ×440.

the presence of *E. corrodens* and possibly other gram-negative organisms. In a practical sense, it is reasonable to expect that *A. israelii* cells that are transported from the mouth to other areas of the body, by whatever means, would be accompanied by other organisms, including gram-negative rods, fusobacteria, and anaerobic bacteria.

Lesions caused by *A. viscosus* were enhanced to a limited extent by the presence of *E. corrodens*. The effect appeared to be temporary, as the lesions were confined to the acute stage of the disease. Although the mixed lesions were still present at the end of the experiment (4 weeks), there was strong evidence that they were resolving. Cell numbers of both species in the lesions were steadily declining over the course of the experiment, and viable organisms had almost disappeared by 4 weeks. The central granule had lost its structural integrity, with no sign of new areas of extension. It was heavily infiltrated by mixed inflammatory cells, with only scattered remnants of the gram-positive filaments remaining. The histological appearance of these mixed lesions at 4 weeks was typical of acute *A. viscosus* lesions in the resolving stage, as described previously (1). Thus, it appears safe to conclude that the mixed *A. viscosus-E. corrodens* infection would not have persisted into the chronic stage. A similar result in which an infection caused by smooth *A. israelii* 12597 was confined to the acute stage even when *E. corrodens* was present was described previously (12).

It is possible that other aspects of *Actinomyces* pathogenicity may be enhanced by the presence of associated gram-negative organisms. However, this was not readily evident from the present experiments, as there was no significant change in the size or appearance of the lesions or in the spread of infection. The histological appearance of the mixed lesions was similar to that previously described for pure-culture actinomycotic lesions in this animal model (1). The characteristic inflammatory response to the lesions was not significantly altered by the presence of the gram-negative organisms. The persistent gram-negative organisms in the chronic lesions were probably located inside the central bacterial granule; however, the basic morphology of this structure did not appear to be different from that in pure-culture lesions. The staining methods used were not adequate to clearly distinguish individual cells of the gram-negative organisms within the lesions, even though culturing had established their presence in high numbers. Thus, it was not possible to describe their distribution inside the granule, particularly in relation to the actinomycotic filaments. Further examination of the mixed lesions by electron microscopy and fluorescent-antibody techniques is in progress.

Holm (10) studied ca. 650 patients with nonfistulated actinomycotic lesions containing anaerobic *Actinomyces* spp. In all cases, the *Actinomyces* spp. were accompanied by other organisms. This author hypothesized that actinomycosis is always a mixed infection in which the presence of both the *Actinomyces* spp. and other microbes is a required condition for development of the infection. It was pointed out that culturing of actinomycotic lesions was not always adequate for demonstrating the presence of the associated gram-negative pathogens (10).

The present study demonstrates that even in the case of mixed experimental lesions of known etiology, the presence of associated gram-negative pathogens is not easy to discern in tissue sections processed by standard histological procedures. This fact, combined with the previously mentioned potential for deficiencies in microbiological culturing techniques, makes it easy to understand why the associated gram-negative pathogens in actinomycosis have often been overlooked.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service grant DE-02847 from the National Institute of Dental Research and in part by a grant from the Colgate-Palmolive Co.

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