PLAQUE FORMATION AND PERIODONTAL PATHOLOGY IN GNOTOBIOTIC RATS INFECTED WITH AN ORAL ACTINOMYCETE

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Certain filamentous bacteria, indigenous to the oral cavity of the hamster, were found to be associated with periodontal disease commonly seen in this animal.[1,2] Similar organisms were isolated from gingival plaques in rats and these strains also induced the disease when inoculated into hamsters.[3] This evidence would seem to indicate an etiologic relationship between these filamentous bacteria and periodontal disease in both the rat and hamster.

Evaluation and interpretation of these experiments is complicated by the presence of other bacteria in the oral environment. The extent to which the filaments may depend on other members of the oral flora in the initiation of disease would be difficult, if not impossible, to assess in conventional animals. For this reason, filamentous bacteria from the rat and hamster were tested in gnotobiotic rats for their ability to induce plaque formation and periodontal disorder in the absence of any other bacteria.

EXPERIMENTAL METHODS

Experiment 1. The purpose of the first gnotobiotic experiment was to test the ability of the hamster filamentous organism to establish itself and to induce periodontal alterations in the germfree rat. Six Fisher rats, 30 days old, from the NIH germfree inbred, breeding stock were housed 2 per wire-bottom cage in an isolator of the type described by Gustafson.[4] They were fed diet 585VC, a high carbohydrate caries-producing diet fortified with vitamins and casein [5] which was steam-sterilized when introduced into the isolator. Sterile, canned tap water was provided *ad libitum.*

The infecting organism used in this experiment was a filament-forming actinomycete (strain T6A) isolated from periodontal plaque in a hamster. This strain of actinomycete had been made resistant to 50 μgm per ml of oxytetracycline·HCl and had been previously used to demonstrate an etiologic relationship between these organisms and periodontal disease in hamsters.[2] Inoculum cultures were grown in narrow-necked glass vials containing Trypticase-soy broth (Baltimore Biological Laboratories, Baltimore, Md.) under an atmosphere of 95 per cent N_2 + 5 per cent CO_2. After 24 hours incubation at 37° C, duplicate vials were heat-sealed and taken into the isolator through a germicidal trap. Each rat was infected orally with a cotton swab soaked in the broth culture. A portion of the broth culture was used to contaminate the drinking water in each cage. The unopened vial was removed from

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the isolator in order to determine the purity and viability of the inoculum. During the course of the experiment the isolator unit was sampled bacteriologically at weekly intervals as a check on extraneous contamination. After it had been determined that the infecting organism was established, the rats were removed from the isolator and sacrificed according to the following schedule: 1 rat at 13 days, 1 rat at 53 days, and 3 rats at 89 days, leaving 1 Fisher rat remaining in the unit. At the time of sacrifice bacteriological samples were taken from the cecum and from plaque deposits on the teeth. The heads were fixed in 10 per cent formalin and split sagittally. One-half of the head was retained for gross examination of the teeth and soft tissues and for notations of plaque formation. The other half was processed for histologic examination of the periodontal tissues. One of the 3 Fisher rats removed from the experiment at 89 days was necropsied and the internal organs examined for gross evidence of infection or other abnormality. Samples of kidney, lung, spleen, liver and blood were inoculated into Trypticase-soy broth and into fluid thioglycollate medium.

When the last Fisher rat had been in the isolator for 106 days, 3 weanling, Sprague-Dawley rats obtained from the NIH germfree breeding colony were introduced into the monocontaminated isolator. For the remainder of the experiment all of the rats were fed diet 2000,[1] a finely powdered, high carbohydrate diet. For use in the gnotobiotic studies the diet was fortified with Gustafsson's vitamin supplement [4] and sterilized by irradiation at 4 megarads in a Van der Graff accelerator. The Sprague-Dawley rats were removed from the isolator and sacrificed at 26, 53 and 62 days, respectively. The last Fisher rat was removed after a total experimental time of 159 days.

Techniques of bacteriologic examination and processing of the heads were the same as described previously. Blood samples were obtained by cardiac puncture from the three Sprague-Dawley rats and the last Fisher rat to be removed. Antibody titers to the infecting organism were determined in the sera of these four animals using essentially the hemagglutination test described by de Araujo, Varah, and Mergenhagen.[6] Antigen for the test was a soluble French press extract of cells grown aerobically in Trypticase-soy broth.

Experiment 2. In the second gnotobiotic experiment a filament-forming organism (strain RF-1) isolated from cervical plaque in a conventional rat [3] was tested for its ability to induce periodontal disorder in germfree Sprague-Dawley rats. Eight weanling rats from NIH germfree stock were housed 2 per cage in a Gustafsson isolator and fed irradiated diet 2000V for the entire experimental period. Details of handling the bacterial culture and the technique of inoculation were the same as in experiment 1.

The rats in experiment 2 were sacrificed at intervals of increasing exposure to the diet and organism, i.e., 1 rat at 32 days, 1 rat at 46 days, 3 rats at 49 days, and 3 rats at 98 days.

As a parallel control to this experiment 6 germfree Sprague-Dawley rats were maintained in a separate isolator. They were fed the same diet and remained germfree throughout the experimental period. These control animals were removed according to the following schedule: 1 rat at 37 days, 1 rat at 39 days, 3 rats at 53 days and 1 rat at 95 days.

After sacrifice all of the animals in experiment 2 and the controls were bled, cultured bacteriologically and the heads fixed using the same procedures as in experiment 1.

RESULTS

The hamster organism, strain T6A, used to infect the Fisher and Sprague-Dawley rats in experiment 1 showed definite evidence of colonization in the oral cavity, around the molar teeth. Restricted areas

of plaque observed were usually confined to 1 or 2 locations in a particular animal. In some cases plaque was seen mainly around the third molars, in other cases along the mesial surface of the first molars. There were no generalized, heavy deposits of plaque in any of the animals such as were seen in conventional hamsters infected with this organism.[2] Plaque formation in all of these animals of experiment 1 was confined to the gingival margins of the molar teeth and did not appear to extend down into the gingival sulcus.

The internal organs in the Fisher rat sacrificed at 89 days appeared normal except for the typically enlarged cecum. No bacterial growth resulted from the incubated tissue samples. Apparently the organism was confined to the alimentary canal.

Monoinfection of Sprague-Dawley rats with the rat oral filament, strain RF1, resulted in a generalized formation of heavy plaque deposits around the molar teeth (Fig. 1). Both the maxillary and mandibular quadrants were involved. The deposits appeared heavier on the buccal surfaces, especially on the maxillary quadrants. Also, an impression was gained that the deposits were heavier in the animals sacrificed early in the experiment. Exact comparisons of plaque formation are difficult since no attempt was made to score or measure the amount of plaque objectively in each animal. The appearance of plaque in one of the monoinfected animals is shown in Figure 1.

The infecting organism was recovered from every animal in both experiments at the time of sacrifice. A sample from the enlarged cecum was always cultured and in most instances plaque samples were also cultured and found to be positive for the filaments. With some of the animals in experiment 1, both cecal and plaque samples were plated out in parallel on blood agar plates containing 1 μgm per ml oxytetracycline HCl and on control blood plates. Strain T6A was always recovered in equal numbers on the two media indicating that it was still drug-resistant.

Molar quadrants which were stripped of soft tissue to reveal alveolar bone levels appeared grossly the same as the uninfected control animals. There was no evidence of excessive bone loss in any of the monoinfected animals from experiments 1 or 2 (Fig. 2).

Antibodies to the infecting organisms in both experiments were present in most of the animals at relatively low titers. There did not appear to be any clear-cut relationship between antibody levels and length of exposure to the infectious agent. In fact, the Fisher rat sacrificed at 159 days had no detectable antibodies. The 3 rats with the highest titers in experiment 2 had all been exposed for 49 days or longer. Data on antibody response in the two experiments are summarized in Table I.

The uninfected Sprague-Dawley control animals were essentially free of any kind of gingival deposits except for some impacted hairs and occasional food deposits in the molar sulci. The soft tissues appeared firm and well-defined. A hard, brownish-black pellicle which may have represented a type of precalculus deposit was seen on the coronal surfaces of some of the molar teeth (Fig. 3).

TABLE I

ANTIBODY TITER IN GNOTOBIOTIC RATS INFECTED WITH FILAMENTOUS ORAL BACTERIA

Experiment 1			*Experiment 2*		
Rat number	Days on experiment	Hemagglutination titer	Rat number	Days on experiment	Hemagglutination titer
SD-1	26	1/40	SD-1	32	1/10
SD-2	53	1/80	SD-2	46	1/10
SD-3	62	1/40	SD-3	49	1/10
F6	159	0	SD-4	49	1/40
Control SD*	—	0	SD-5	49	1/40
Control F*	—	0	SD-6	98	1/20
			SD-7	98	1/10
			SD-8	98	1/40
			Control S.D.*	—	0

* Control sera from uninfected germfree Sprague-Dawley and Fisher rats.

Histologic Features. The periodontal structures in 8 rats, 3 from each experiment and 2 germfree controls, were examined histopathologically to assess the degree of periodontal involvement. Molar quadrants from half of each of the 8 heads were serially sectioned (one quadrant from each half sectioned mesiodistally and the other sectioned buccolingually). Alternate sections were stained with hematoxylin and eosin and with the Brown and Brenn method. Abnormalities in each section were described and recorded and the severity of the alterations tabulated by the number of sections described (Table II). The greater the number of sections showing periodontal changes or the greater degree of destruction of the interdental papilla, the higher the classification of periodontal involvement (minimal, moderate, severe) that was assigned.

The Fisher rats from experiment 1 showed inflammation which could be considered minimal. A few inflammatory cells could be seen infiltrating the intact but slightly edematous epithelium. The capillaries in the connective tissue were hyperemic but presented no increase in circulating inflammatory cells. Plaque deposits were observed on top of gingival tissue without compression of the soft tissues. The Brown and Brenn stain revealed that no organisms had invaded the soft tissues.

The Sprague-Dawley rat from experiment 1 revealed evidence of

damage to the interdental papillae with exudate escaping from the injured papillae and mixed with the plaques (Fig. 4). Impacted and embedded hairs were commonly found. A single carious lesion was found in this animal and the infecting organisms were encountered deep in the dentinal tubules (Fig. 5). This was the only carious lesion detected in all of the animals examined. Compression of gingival tissue associated with the presence of plaque was seen in buccolingual sections. Epithelial migration along the curvature of the root was also observed.

In the Sprague-Dawley rats examined from experiment 2, severe destruction of the gingival soft tissues was associated with large purulent plaques. The characteristics of interdental papillary loss, epithelial migration and proliferation, exudation and embedded hairs were pronounced (Fig. 6). Focal bone resorption was found in one interproximal area of one animal. In Figure 7 it can be observed that despite gross

TABLE II

PERIODONTAL ABNORMALITIES IN GNOTOBIOTIC RATS INFECTED WITH FILAMENTOUS ORAL BACTERIA

Animal no.	Rat strain	Infected with	Diet	Age (days)	Days on experiment	No. of sections examined	Degree of periodontal involvement
			Experiment 1				
F-2	Fisher	T6A	585VC	83	53	7	Minimal
SD-2	Sprague-Dawley	T6A	2000V	83	53	19	Moderate
F-6	Fisher	T6A	585VC + 2000V	189	159	9	Minimal
			Experiment 2				
SD-1	Sprague-Dawley	RF1	2000V	62	32	27	Severe
SD-2	"	"	"	79	49	23	"
SD-3	"	"	"	128	98	20	Moderate
SD-C	"	Uninfected control	"	67	37	7	Minimal
SD-C	"	"	"	83	53	2	Very minimal

tissue destruction no bacteria were located within the soft tissue, only in the exudate. Large bacterial plaques bordered by exudate were observed in the gingival craters. Micro-organisms were found within the tissues only when carried along with embedded hairs.

Very minimal gingival changes were noted in the germfree control animals. A few impacted and embedded hairs were found but there was no significant degree of inflammation. All of the periodontal structures were intact.

Discussion

These experiments confirm observations made in the hamster[2] that oral infection with specific filament-forming organisms from the hamster or rat induces cervical plaque formation and periodontal pathologic alterations. A similar disease picture is commonly seen in conventional rats fed high carbohydrate, soft diets. Plaque accumulations and gingival alterations in conventional Sprague-Dawley rats were described by König and Mühlemann.[7] Minimal changes in the gingivae reported by those authors could be attributed to the relatively short experimental period. The present work indicates that the oral filament-forming organisms can produce gingival abnormality to a certain extent in the absence of other bacteria.

The single carious lesion seen in a rat infected with the hamster filament may have resulted from the organism gaining access to the dentin through a pre-existing defect in the enamel. Even though Hurst, Nuckolls, Frisbie and Marshall[8] had reported that oral actinomycetes invaded excised, unerupted hamster molars *in vitro*, we have not observed the penetration of intact mature enamel by these organisms *in vivo*. Lesions in the exposed dentin of hamster root surfaces have been found to be associated with gingival plaques in animals infected with these filaments.[1] Root surface lesions were not seen in the rats used in the present gnotobiotic experiments.

Evidence to date on experimental periodontal disease indicates that the primary role of filamentous bacteria may be to initiate plaque deposition and pocket formation. There is no indication from this or previous work that this type of organism invades the soft tissue. In fact, tests of strain T6 for hyaluronidase activity by the method of Tolksdorf, McCready, McCullagh and Schwenk[9] were negative.

Histologic examination of the periodontal structures in the monoinfected rats has indicated a minimal degree of focal alveolar bone loss in comparison to uninfected control animals. However, there was certainly no evidence of the excessive bone loss which may be seen in conventional hamsters or rats on similar diets. The severity of the disease would therefore seem to be less in the monoinfected than in conventional animals but direct quantitative comparisons have yet to be made. To what degree other oral bacteria would have increased the extent of inflammation and bone resorption is not known but the application of gnotobiotic techniques would seem to offer a most practicable method to explore this problem.

Summary

Aerobic actinomycetes isolated from hamsters and rats with periodontal infections were used to monoinfect germfree Sprague-Dawley

and Fisher rats maintained on high sugar diets. Colonization in the oral cavity occurred with bacterial plaque formation around the molar teeth. Pathologic alterations of the periodontal tissues, including focal bone loss, were revealed in histologic sections. There was no indication that the infecting organisms were able to invade the soft tissues. Germfree control animals fed the same diet were essentially free of the periodontal abnormality observed in the monoinfected animals except for lesions associated with impacted hairs.

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[*Illustrations follow*]

Legends for Figures

FIG. 1. Bacterial plaque on the teeth of a Sprague-Dawley rat monoinfected with a filamentous organism (strain RF-1). The plaque was stained with safranin for contrast.

FIG. 2. Maxillary molar quadrant in a Sprague-Dawley rat monoinfected with a filamentous organism (strain RF-1).

FIG. 3. Normal molar quadrant of a germfree rat showing a dark-stained deposit.

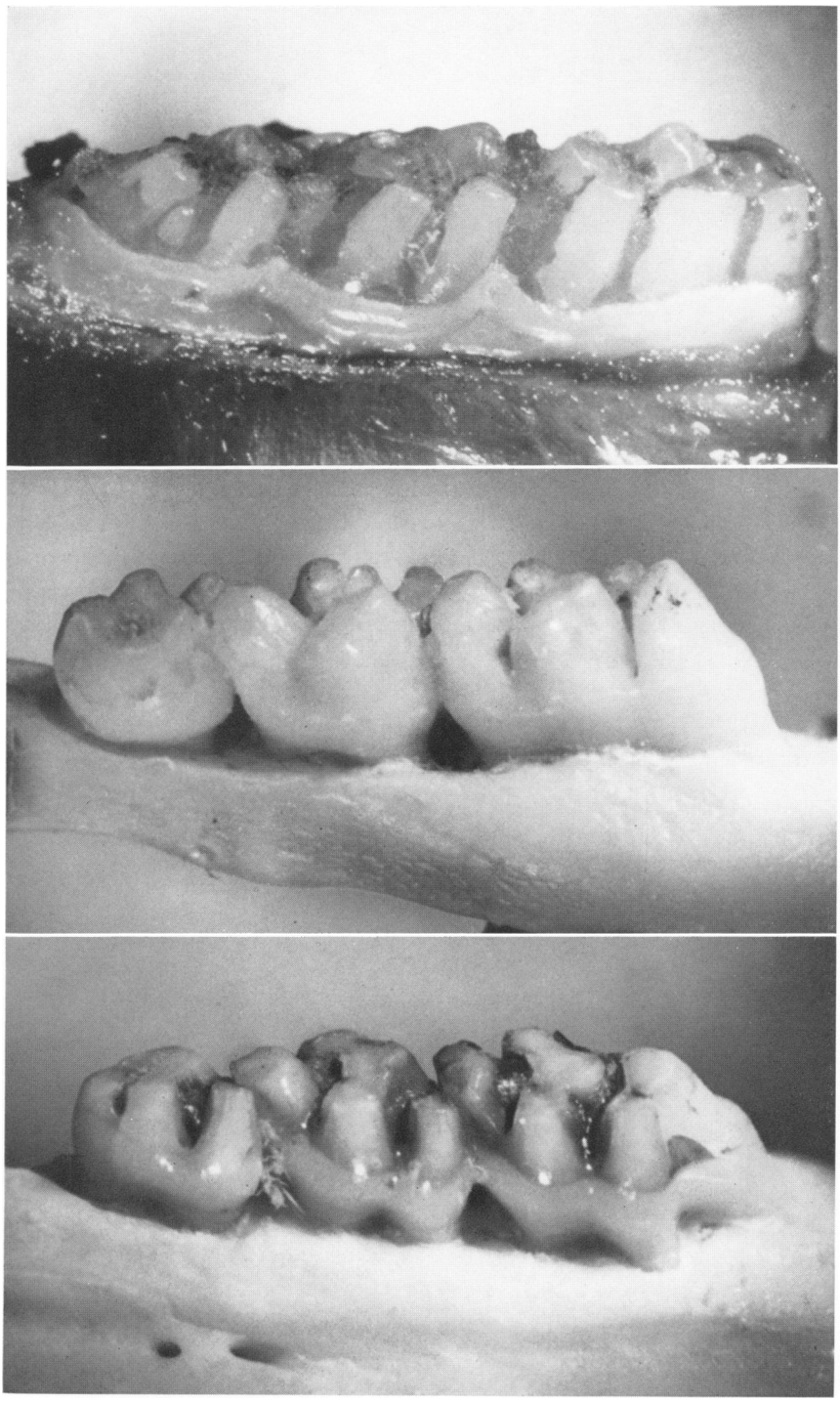

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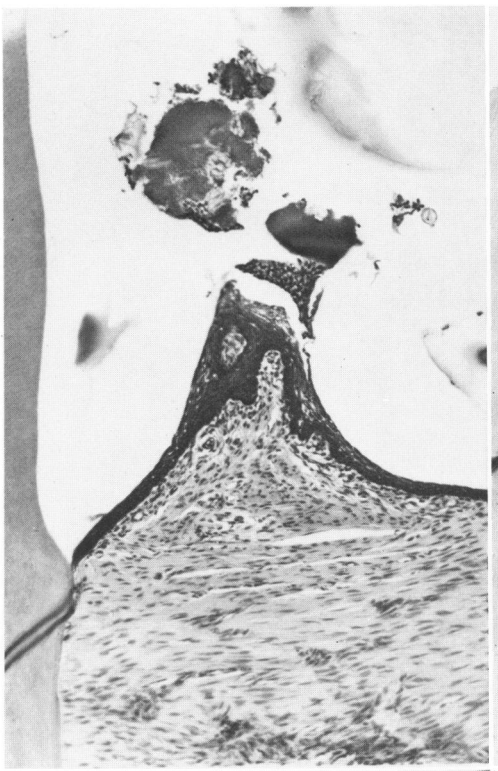
FIG. 4. Sprague-Dawley rat in experiment 1. The interdental papilla has been damaged by the plaque and exudate has mixed with the plaque. Hematoxylin and eosin stain. × 100.

FIG. 5. Caries-like lesion in a monoinfected rat. Infecting organisms have extended deeply into the dentinal tubules. Brown and Brenn stain. × 100.

FIG. 6. Sprague-Dawley rat from experiment 1. A large plaque is associated with loss of papillary architecture, migration and proliferation of epithelium and embedded hairs. Hematoxylin and eosin stain. × 100.

FIG. 7. There is gross tissue destruction with organisms confined to the plaque and absent from soft tissues. Brown and Brenn stain. × 100.



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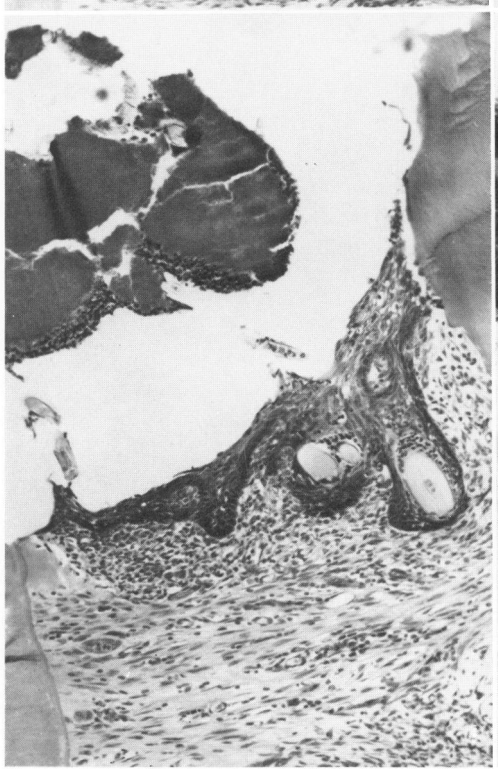

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