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Localization of Rate-Limiting Barrier to Penetration of Endotoxin Through Nonkeratinized Oral Mucosa In Vitro

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The penetration of tritiated bacterial endotoxin through nonkeratinized oral mucosal epithelium was studied using an in vitro model system and radioautographic tracer techniques. The basement membrane region of the epithelium was the rate-limiting barrier to penetration, and this barrier effect was independent of the direction and duration of penetration as well as any clearance effects of the vasculature.

It is generally accepted that toxic, antigenic, or enzymatic by-products of bacterial cells are important mediators in the initiation of inflammatory periodontal disease.^{1,2} Therefore, the ability of the gingival sulcular epithelium to minimize the penetration of these bacterial by-products into the underlying connective tissue represents a primary mechanism of host defense.

Endotoxin, the lipopolysaccharide component of the cell walls of gram-negative bacteria, has been implicated in the origin of inflammatory periodontal disease by numerous investigations.³⁻⁹ In this regard the finding of Schwartz, Stinson, and Parker's¹⁰ that endotoxin may penetrate the intact, nonkeratinized, stratified squamous epithelium of the gingival sulcus, suggests that endotoxin may be important not only in the progression but also in the initiation of inflammatory periodontal disease. Moreover,

recent reports that nutritional deficiency can significantly increase the penetration of bacterial endotoxin through nonkeratinized oral mucosa¹¹⁻¹³ indicate a potential mechanism by which nutritional deficiency may predispose the host to inflammatory periodontal disease.

In an attempt to more clearly define the mechanism by which nutritional inadequacy alters the ability of endotoxin to permeate oral mucosal epithelium, this investigation was designed to localize the epithelial structure that represents the rate-limiting barrier to penetration. Although Schwartz, Stinson, and Parker¹⁰ noted that the penetrating endotoxin appeared to "build-up" at the basement membrane region of the epithelium, their study was not specifically designed to investigate this problem, and this observation was a qualitative one. In addition, using an in vivo system to study this problem makes it difficult to evaluate penetration because once the material enters the connective tissue, it may be rapidly carried away by the blood supply. In order to eliminate this potential vascular "wash-out," the present investigation was conducted in vitro.

Materials and Methods

Escherichia coli endotoxin Type 055:B5^a was gas tritiated^b according to the method described by Schwartz, Stinson, and Parker,¹⁰ and free tritium was removed from the endotoxin by exhaustive dialysis against distilled water as described by Schrader and Woolfrey.¹⁴ The final specific activity of the tritiated endotoxin was 94.5 $\mu\text{Ci}/\text{mg}$.

The epithelium of the ventral surface of

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^a Difco Laboratories, Detroit, Mich.

^b New England Nuclear Corp., Boston, Mass.

the base of the guinea pig's tongue has been shown to be histologically analagous to human gingival sulcular epithelium.¹¹ In addition, this tissue has been used as a model in previous studies on nutritional alterations of the oral mucosal epithelium's permeability.¹²⁻¹³ Therefore, full-thickness mucosal explants from the ventral surface of the base of the guinea pig's tongue were used in this study. Two mucosal explants (approximately 4 mm²) were dissected from the tongues of each of eight, young adult, male Hartley guinea pigs.^c These mucosal explants were placed on sterilized filters of 0.3-micrometer (μ m) porosity,^d which were suspended in plastic culture dishes over Dulbecco's modification of Eagle's medium.^e This medium was supplemented with guinea pig serum (20% v/v), L-ascorbic acid (50 μ g/ml), L-proline (100 μ g/ml), penicillin (100 units/ml), and streptomycin (100 μ g/ml). Six of the explants were oriented normally, that is, placed with the connective tissue surface on the filter, and six explants were placed on the filters inverted, that is, epithelial surface down.

A 1- μ l volume of tritiated endotoxin solution (0.945 μ Ci) was placed on the center of each of the aforementioned 12 experimental

tissue preparations. Two normally oriented and two inverted explants, without endotoxin added, served as controls. One half of the preparations were incubated for two hours, and the remaining half for six hours in a humidified atmosphere of 95% air and 5% CO₂ at 37 C.

After the appropriate incubation periods, the tissues were removed from the culture, washed for two minutes in two 5.0-ml changes of phosphate buffered saline, oriented on O.C.T. Compound^f and frozen on solid CO₂. The frozen blocks were sectioned at 6.0 μ m in a cryostat according to the method of Courant and Bader¹⁵ to minimize section contamination. The sections were fixed and processed for radioautography as described by Schwartz, Stinson, and Parker,¹⁰ and the latent image was allowed to develop for four weeks at 4.0 C. The radioautographs were stained with toluidine blue.¹⁶ Untreated control tissues, as well as blank slides, were processed by a method identical to the one used for the experimental tissues.

A grain count analysis was conducted based on the premise that, in a radioautograph of a radiolabeled penetrant, the silver grains will appear to back up at the structure which limits the movement of the penetrant through the tissue. Using an ocular grid, the number of grains in one, 400- μ m² field in the

^c Elm Hill Farm, Chelmsford, Mass.

^d No. PHWP 04700, Millipore Corp., Bedford, Mass.

^e Gibco, Grand Island NY.

^f Ames Co., Elkhart, Ind.

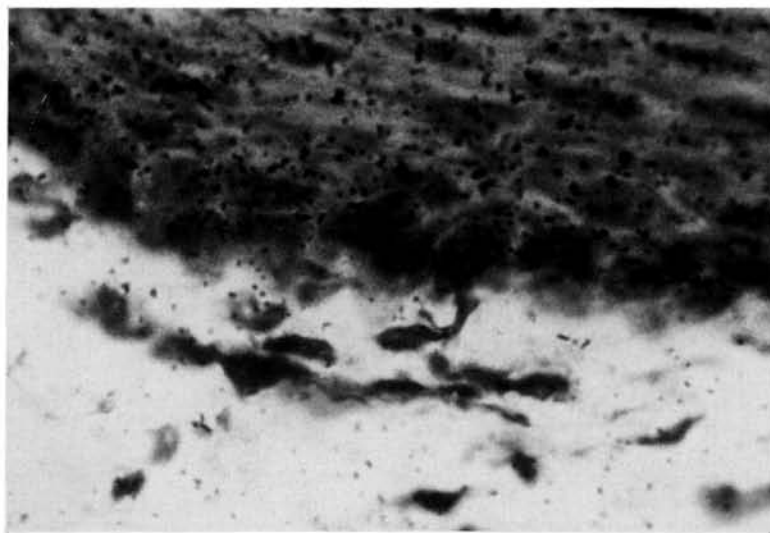


FIG 1.—Radioautograph of ³H-endotoxin penetration through mucosal explant from epithelium to connective tissue (toluidine blue stain; mag 1,800 $\times$; six hours).

epithelium approximating the basement membrane was compared with the grain count from the adjacent 400- μm^2 field in the connective tissue. Five of these determinations were made randomly for each tissue preparation, and the means of each group were compared using Student's *t* test. In addition, "barrier ratios"—the ratio of the number of grains on the penetrating side of the basement membrane to the nonpenetrating side—were calculated for each group of explants.

Results

As summarized in the table, the mean grain counts for the fields on either side of the basement membrane were significantly different for each of the experimental groups. In addition, the barrier ratios were all greater than unity indicating that in all experimental groups the number of grains was significantly greater on the side of the basement membrane to which the tritiated endotoxin was applied. Thus, when the radiolabeled endotoxin was applied to the epithelial surface of the normally oriented explants, the silver grains accumulated on the epithelial side of the basement membrane (Fig 1). In contrast, when the endotoxin was placed on the connective tissue surface of the inverted explants, the grains backed up on the connective tissue side of the basement membrane (Fig 2). Therefore, it is apparent that, regardless of the direction or duration of penetration, the basement membrane region demonstrates a considerable ability to minimize the penetration of bacterial endotoxin through this nonkeratinized mucosal tissue. Comprehensive examinations of the radioautographs indicated that there were no other structures in this tissue that demonstrated a barrier effect comparable to the basement membrane.

Analysis of blank slides demonstrated a low background level of 1.1 ± 0.8 (SD) grains per 100 μm^2 . The fact that counts on control slides were not significantly greater than the background levels indicates that minimal contamination occurred during the preparation of the slides.

Discussion

Barrier function is the property of epithelial surfaces that minimizes passage of noxious substances from the outer epithelial surface to the underlying connective tissues.

Therefore, in many infectious processes the epithelial barrier represents the primary site of bodily defense mechanisms. Although active processes, such as phagocytosis by epithelial cells¹⁷⁻¹⁸ may be involved in maintaining the epithelial barrier, the overall effectiveness of the barrier is generally considered to be passive in nature.¹⁹⁻²⁰ This conclusion is based on the fact that epithelial tissues refrigerated for up to 48 hours or frozen for several days show little difference in barrier function when compared either to freshly isolated tissues or to the epithelium *in vivo*.²⁰⁻²² Another factor that supports the passive nature of the epithelial barrier is that Fick's law, which states that the speed of penetration of a substance through a membrane is proportional to the concentration gradient, holds true in most studies of epithelial penetration.²⁰ Based on the passive nature of the epithelial barrier as just discussed, as well as the fact that the organ culture system used in the present study can maintain the viability of mucosal explants

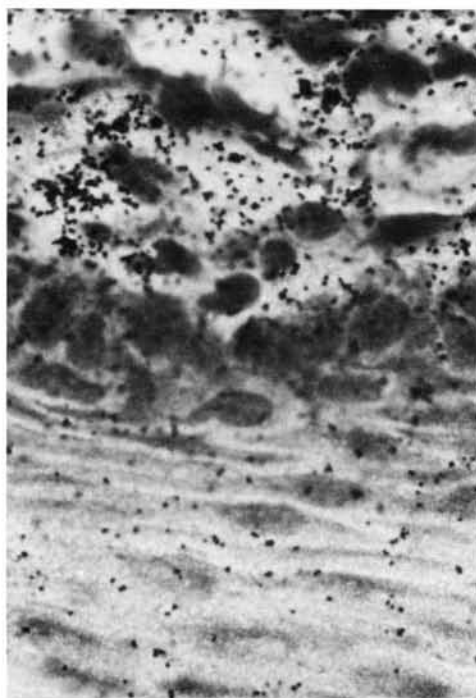


FIG 2.—Radioautograph of ^3H -endotoxin penetration through mucosal explant from connective tissue to epithelium (toluidine blue stain; mag 1,800 $\times$; six hours).

for up to two days,¹¹ it is probable that the model system used in this study accurately reflects the barrier function of the mucosa in vivo.

There are a considerable number of reports that suggest that the basement membrane may be the limiting barrier in macromolecular penetration of nonkeratinized mucosal surfaces.^{10,22-28} Indeed, the present study of epithelial barrier function supports this conclusion. In addition, the present investigation has expanded the current body of information about the barrier function of basement membranes in several ways.

First, since the investigation was conducted in vitro, it was not subject to any effects of blood or lymph circulation that may remove the endotoxin rapidly once it enters the connective tissue. This rapid removal from the connective tissue, or "wash-out" effect, could give the false appearance in the in vivo studies that the passage of endotoxin into the connective tissue was blocked by the basement membrane. It is important to note that this wash-out effect is apparently very large. Using a radioautograph from the report of Schwartz, Stinson, and Parker¹⁰ on the penetration of tritiated endotoxin through the gingival sulcus in vivo, one can calculate a barrier ratio at the basement membrane of 17:1. In contrast, the present in vitro investigation using an identical tracer molecule and a similar tissue demonstrated a maximal barrier ratio at the basement membrane of 2.2:1 (Table). It is probable that the difference in barrier ratio

between the in vivo and in vitro situation is in a large part, due to the clearance of the vasculature. In any case, it is clear that the effects of the vasculature cannot be ignored in studies of epithelial barrier function.

The study of penetration across the basement membrane in both directions also provided useful information. The finding that the barrier effect of mucosal epithelial basement membrane is independent of the direction of penetration (Table) suggests that the barrier effect is primarily a function of the physicochemical state of the membrane itself and is not necessarily dependent on the activity of cellular components on either side. Finally, the fact that the barrier effect appears to be independent of the duration of penetration (Table) strongly suggests that this effect is a function of the basement membrane and not merely a manifestation of a diffusion "front" of molecules.

Based on this investigation as well as the evidence in the literature, it is reasonable to state that the basement membrane is the probable rate-limiting barrier to the penetration of macromolecular endotoxin through nonkeratinized stratified squamous epithelium. The significance of this finding is particularly evident in those diseases, such as gingivitis or scarlet fever, in which only toxic by-products from bacteria, and not the bacteria themselves, need penetrate the epithelial barrier to initiate the disease process. Since a great many of these bacterial by-products are macromolecules, the importance

TABLE
GRAIN COUNT ANALYSIS OF TRITIATED ENDOTOXIN PENETRATION IN VITRO
THROUGH NONKERATINIZED GUINEA PIG ORAL MUCOSA

Tissue	Incubation (hrs)	Mean No. of Grains $\pm$ SD in Adjacent 400- μ m Fields*			
		Epithelium	Connective Tissue	Barrier Ratio†	P‡
Control	6	6.8 $\pm$ 4.6	6.5 $\pm$ 4.3	. . .	>0.05
Normal	2	17.3 $\pm$ 5.3	8.8 $\pm$ 4.8	2.0/1.0	<0.01
Normal	6	53.5 $\pm$ 10.2	33.6 $\pm$ 5.3	1.6/1.0	<0.001
Inverted	2	53.7 $\pm$ 10.3	86.6 $\pm$ 10.5	1.6/1.0	<0.001
Inverted	6	39.6 $\pm$ 11.4	85.5 $\pm$ 21.0	2.2/1.0	<0.001

* The mean grain count for each group represents the average of 15 fields from three different preparations.

† Barrier ratio = $\frac{\text{No. of grains in 400-}\mu\text{m}^2 \text{ field on side of basement membrane endotoxin solution was applied}}{\text{No. of grains in adjacent 400-}\mu\text{m}^2 \text{ field on opposite side of basement membrane}}$

‡ As determined by Student's *t* test.

of the basement membrane barrier function is readily apparent. The fact, as calculated from the data in the table, that the basement membrane can restrict macromolecular penetration into the connective tissue by an average of almost 50% indicates that a loss of this barrier effect would nearly double the antigenic or toxic challenge to the connective tissue. Therefore, it is clear that the basement membrane represents an important component of the host's barrier defense, and local, hormonal, or nutritional^{29,30} factors that may affect the function of this barrier are worthy of further investigation.

It is interesting to note that these results support a recent hypothesis³¹ that a very early or preinflammatory flow of gingival fluid may be osmotically mediated. That is, before the initiation of classical inflammatory gingival exudation, macromolecular by-products of the bacterial plaque may accumulate at the basement membrane to generate an osmotic gradient. This gradient may result in a temporary flow of gingival fluid that is not inflammatory in origin. The possibility that gingival fluid may be derived by two distinct mechanisms, osmosis and inflammation, can explain many of the confusing properties of this fluid.³¹

Conclusions

The basement membrane of nonkeratinized oral mucosal epithelium from the guinea pig is the probable rate-limiting barrier to the penetration of tritiated endotoxin. This barrier effect of the basement membrane is independent of the direction and duration of penetration as well as any clearance effects of the vasculature. Therefore, factors such as nutritional deficiency or bacterial collagenase that can alter the integrity of the basement membrane may predispose the host to infectious disease.

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