

Lysozyme Activity in the Serum, Saliva and Tears of Germfree and Conventional Rats and Mice

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The germfree animal, when compared to its conventionally-reared counterpart, has been characterized as having generally underdeveloped defense mechanisms: e.g. underdeveloped lymph nodes with no or very rare reaction centers, slightly reduced numbers of circulating leucocytes, reduced number of scattered reticuloendothelial elements in the ileum wall, low gamma globulin levels and absence of most of the circulating antibacterial antibodies found in the serum of conventional animals (10, 16).

Lysozyme was investigated as one of the factors involved in the non-specific defense mechanisms of the host. The presence of substantial quantities of lysozyme in the serum, saliva and tears of a number of mammalian species has been reviewed by Afonsky (1) and reported by various investigators (2, 3, 5, 6, 7, 8, 11, 13, 15). It was of interest to determine whether the level of lysozyme would be different in germfree animals in the absence of stimulation from a viable associated microflora. Some investigators (4, 12) have considered the possibility that lysozyme may help protect oral tissues from infection by lysing or otherwise inhibiting the enzyme-sensitive microorganisms which gain access to the mouth. Lysozyme could be a factor which limits the indigenous oral flora to lysozyme-insensitive strains. Gibbons *et al.* (9) have surveyed the numerically most prominent bacteria indigenous to the oral cavity of man for their susceptibility to lytic action of lysozyme. None of 112 pure bacterial isolates, representing 13 major groups of the human cultivable oral flora, were lysed by high levels of lysozyme nor did the enzyme at 50 mcg/ml inhibit their growth *in vitro*. Lysozyme-sensitive *Micrococcus lysodeikticus* on the other hand was inhibited by 0.5 mcg/ml, the lowest level reported. It thus seems that the oral flora is limited to lysozyme resistant organisms.

In the present study, lysozyme was not detected by *in vitro* assays on the saliva of germfree rats and mice. It seems significant that *M. lysodeikticus* could be recovered from the oral cavity of gnotobiotic rats inoculated orally about 2 weeks earlier with the mentioned microorganism. The *in vivo* test thus supports the negative salivary lysozyme levels found by *in vitro* methods. This study also revealed a relationship between the presence of salivary leucocytes and lysozyme which suggested that salivary lysozyme may be of local leucocyte origin.

Materials and Methods

The germfree animals used were taken from the animal colonies maintained at Lobund Laboratory. Bacteria-associated gnotobiotic

1. This investigation was supported in part by U. S. Public Health Service grant DE-01887 from the National Institute of Dental Research and in part by the University of Notre Dame.

animals were derived from gnotobiotic germfree stock by oral inoculation with known microorganisms and holding them in gnotobiotic state for the desired period of time. Conventionalized animals were animals that were once germfree but were brought out into the external environment, inoculated orally with a fecal suspension from conventional animals, and maintained thereafter in the conventional animal room. Conventional controls were animals which were born and reared in the open animal room by conventionally-reared parents.

Flow of saliva and tears was stimulated by subcutaneously injected methacholine (0.5 mg/kg body weight) after the animals were anaesthetized with intraperitoneal administration of Nembutal (25 mg/kg body weight).

Saliva samples were assayed for lysozyme individually or pooled depending on the volumes collected. Tears were pooled for each group of animals, diluted five times with phosphate buffer (pH 6.2) and centrifuged at 2000 rpm for ten minutes to sediment the red pigment granules. Serum was obtained from blood collected by heart puncture.

The assay of lysozyme was based on the lysis of a *Micrococcus lysodeikticus* suspension by the method of Smolelis and Hartsell (14). Varying dilutions of each of the unknown samples were prepared in volumes of 1.0 ml. Three milliliters of a standard suspension of lyophilized *M. lysodeikticus* substrate (Difco) and 2 ml of pH 6.2 buffer were added to each dilution. The mixtures were incubated at 25°C for exactly 20 minutes. The initial and the final absorbance were read at 450 millimicrons using a Spectronic 20 spectrophotometer (Bausch and Lomb Inc.). Lysozyme levels were interpolated from a standard curve using crystalline egg-white lysozyme (Difco).

Total leucocyte counts were determined in a hemocytometer immediately following sample collection. Differential leucocyte counts were made from smears stained with Wright or Giemsa stains.

Results and Discussion

Lysozyme levels in the serum, saliva and tears of germfree and conventionally-reared rats and mice are reported in Table 1. There was no difference between the germfree and conventional rats with regard to the respective lysozyme levels observed in serum and in tears. However, there was a striking difference in the salivary levels. No lysozyme was detected in the saliva of germfree rats while substantial amounts of lysozyme were detected in saliva from the conventionals. The same salivary difference was observed between germfree and conventional CFW mice. Absence of salivary lysozyme in other germfree rodents was further confirmed in germfree Sprague-Dawley rats and C3H mice.

Mucins are known to inhibit lysozyme by forming mucin-lysozyme complexes (13). The possibility existed that the absence of lysozyme activity in germfree animal saliva could be due to complexing, particularly since mucinolytic bacteria were not present under germfree conditions. Simmons (13) reported that "Cetab" (cetyl trimethyl benzyl ammonium chloride) can efficiently dissociate mucin from protein com-

plexes and precipitate the mucin from saliva. Thus "Cetab" was able to increase the lytic activity of saliva by freeing complexed lysozyme.

TABLE 1. Lysozyme levels in serum, saliva and tears of rats and mice.

Category	No. Animals	Lysozyme levels (mcg/ml)		
		Serum	Saliva	Tears
Conventional Lobund Wistar Rats	8	9.2 (8.0-10.0)	8.2 (8.0-9.9)	6.5 (6.3-6.7)
Germfree Lobund Wistar Rats	10	8.6 (6.8-9.9)	0	6.3 (5.0-7.0)
Conventional CFW Mice	50	13.1 (Pool)	2.5	not run
Germfree CFW Mice	50	9.8 (Pool)	0	not run

Germfree Sprague-Dawley rats and C3H mice gave results similar to germfree Wistar rats and germfree CFW mice respectively.

"Cetab" added at 0.2% in the lysozyme assay system employed in the present experiments did not alter the lysozyme levels; germfree saliva remained negative.

The similar serum lysozyme activity in germfree and conventional animals suggested that salivary lysozyme was not derived from the secretion of serum lysozyme through the salivary glands.

Several reports have indicated that much of the lysozyme found in body fluids is of leucocyte origin (3, 5, 6, 15). In the light of the findings in Table 1, the levels of lysozyme and leucocytes in the saliva of germfree and conventional animals were compared in Table 2. The data show that conventional rats have a substantial number of salivary leucocytes accompanied by appreciable lysozyme levels. The germfree rats on the other hand displayed no salivary leucocytes and no salivary lysozyme. These observations lend supportive evidence that oral leucocytes may be the source of salivary lysozyme, especially since the serum lysozyme levels were not reflected in the salivary secretions of the germfree group.

Since salivary lysozyme was absent in the absence of a viable flora, salivary lysozyme levels were assayed in animals brought into gnotobiotic mono-association with selected pure cultures of bacteria. The results are recorded in Table 3. The data show that mono-association of germfree rats with *Streptococcus faecalis*, *Micrococcus lysodeikticus* or two groups with *Lactobacillus casei* had virtually no effect on increasing the lysozyme levels or salivary leucocyte counts of these animals. Similarly, two groups of rats accidentally contaminated with *Sarcina* sp. or *Micrococcus* sp. respectively gave similar negative results. These organisms could be readily isolated from the mouth of these animals but they apparently lacked leucotactic activity in the oral cavity. It is

TABLE 2. Lysozyme levels and leucocyte counts in germfree and conventional Lobund Wistar rats.

Category	No. Rats	Lysozyme mcg/ml		Salivary Leucocytes No./mm ³			
		Serum	Saliva	Tears	Total	Polymorphs	Monocytes Lymphocytes
Conventional	5	8.2 (7.3-11.4)	9.4 (8.0-12.1)	6.5 (pool)	10,796 (960-31,200)	5,695 (403-6521)	5,416 (326-21,840)
Germfree	15	8.6 (6.1-9.5)	0	6.0 (pool)	0	0	1,664 (130-3120) 0

TABLE 3. Lysozyme levels in serum, saliva and tears and salivary leucocytes in gnotobiotic rats mono-associated with pure cultures of bacteria.

Category		Salivary Leucocytes per mm ³	Serum	Saliva	Tears
Mono-Streptococcus faecalis (150 days)	4	not run	15.0 (pool)	<1	not run
Mono-Lactobacillus casei (200 days)	4	not run	7.3 (6.6-8.6)	<1	6.6
Mono-Lactobacillus casei (200 days)	3	<1	10.6 (10.4-11.0)	<1	5.0 (pool)
*Mono-Micrococcus lysodeikticus (13 days)	12	0	10.5 (10.0-11.0)	0	not run

* Mono-association with *Sarcina* species or *Micrococcus* species gave similar results.

noteworthy to mention that the highly lysozyme sensitive *M. lysodeikticus* could be isolated from the mouth of the gnotobiotic rat presumably because of the absence of salivary lysozyme in these animals.

This study was extended to rats which were brought into association with more than one bacterial species. The results are summarized in Table 4. One group designated as the "hexa" group, consisted of gnotobiotic rats which had been associated with six known microorganisms: *Lactobacillus casei*, *Streptococcus faecalis*, *Aerobacter aerogenes*, *Staphylococcus epidermidis*, *Bacteroides thetaiotaomicron* and a yeast. All strains were originally isolated from the intestinal tract of conventional rats in the Lobund colony and were selected as non-pathogenic representatives of microbial groups which predominate in the rodent intestine. These animals displayed lysozyme activity of less than 1 microgram/ml. of saliva.

Another group of animals was made up of ex-germfree rats, conventionalized by oral inoculation with a suspension of feces obtained from conventionally reared rats. Two rats sacrificed after 42 days of conventionalization had salivary lysozyme activity of less than one microgram per ml. The animals held in a conventionalized state for 114 days as well as their progeny displayed a slight increase in both salivary lysozyme and leucocytes above the levels obtained in gnotobiotic rats. However, both these groups failed to display the full salivary lysozyme levels found in conventionally-reared rats with no previous germfree experience. No microbiological studies were made to determine whether a "normal" resident oral flora had been established by conventionalization of germfree rats with fecal suspensions from conventional animals. One may speculate that the method of conventionalization may have omitted possible highly leucocytotoxic organisms from the oral flora, but this is pure conjecture at this time.

Summary

Lysozyme levels in serum, saliva and tears of germfree, gnotobiotic, conventionalized as well as conventionally-reared rats and mice were studied. The results showed that lysozyme levels in serum and tears were quite similar in these groups. However, the substantial salivary lysozyme levels found in conventional rats and mice were absent in germfree stock. Similarly, little or no salivary lysozyme was detected in animals derived from germfree stock and either brought into mono-association with various bacterial strains or poly-associated with a six-membered flora obtained from conventional animals. Lysozyme activity in saliva was only partly restored when germfree rats were conventionalized.

The absence of salivary lysozyme corresponded to the absence of salivary leucocytes. Conventional animals showing high salivary leucocytes level also displayed substantial salivary lysozyme.

These observations give further evidence that oral leucocytes may be the source of salivary lysozyme.

The ability of lysozyme-sensitive *M. lysodeikticus* to establish itself in the oral cavity of the rat under gnotobiotic conditions supports the

TABLE 4. Number of salivary leucocytes and lysozyme levels in the serum and saliva of "Hexa-Gnotobiotic"* and conventional rats

Category	No. Rats	Leucocytes/mm ³					Lysozyme (mcg/ml)	
		Total	Polymorphs.	Monocytes	Lymphocytes	Serum	Saliva	
"Hexa" gnotobiotic rats	2					9.25 (9.0-9.5)	<1	
Conventionalized rats (42 days)	2					9.1 (7.3-11.0)	<1	
(114 days)	3	688 (43-1,540)	270 (27-760)	119 (8-262)	218 (8-267)	8.1 (8.0-8.5)	3.3 (1.5-6.3)	
2nd generation	7	259 (<1-484)	139 (<1-296)	25 (<1-59)	67 (<1-105)	9.3 (9.2-9.5)	2.65 (<1-5.2)	

* See text.

in vitro evidence that saliva from germfree animals lacks lysozyme activity.

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