

THE ANTISTREPTOCOCCAL PROPERTY OF MILK

III. THE ROLE OF LACTENIN IN MILK-BORNE EPIDEMICS. THE IN VIVO ACTION OF LACTENIN

BY ARMINE T. WILSON, M.D., AND HERMAN ROSENBLUM, M.D.

(From the Alfred I. du Pont Institute of the Nemours Foundation, Wilmington)

(Received for publication, September 4, 1951)

Because milk-borne streptococcal epidemics have received so much emphasis, there is a tendency in many quarters to believe the antistreptococcal substance in milk can have no practical significance. In this paper the importance of lactenin in accounting for some of the characteristics of milk-borne epidemics will be considered. A discussion will also be given of attempts to protect mice from streptococcal infections by the administration of lactenin.

EXPERIMENTAL

The Role of Lactenin in Milk-Borne Epidemics

Perhaps the clearest definition of the part played by lactenin in milk-borne streptococcal epidemics has been given by Pullinger and Kemp (1). They demonstrated that milk which was collected as cleanly as possible, to minimize bacterial contamination, inhibited the growth of *Streptococcus pyogenes* for 48 to 72 hours as a result of the antistreptococcal substance in the milk. They also studied the fate of streptococci inoculated into pasteurized and raw bottled milk from commercial sources, which contained the normal bacterial flora of such milk, and which was kept at temperatures of 15°, 18° and 22° C. in an attempt to duplicate the conditions of milk handling which occur under normal commercial and domestic circumstances. They found that souring occurred too rapidly for *S. pyogenes* ever to multiply after artificial contamination. They concluded that milk-borne epidemics could rarely occur as a result of direct contamination of the milk after milking, because proliferation was prevented by the antistreptococcal property of the milk, by the natural reluctance of streptococci to multiply at low storage temperatures, and by the readiness with which the saprophytic bacteria grew, rapidly producing a degree of acidity incompatible with growth of *S. pyogenes*. They reviewed a number of streptococcal epidemics in which the source had been traced to bovine mastitis due to *S. pyogenes*, and concluded that mastitis was the usual source of streptococci in milk which caused streptococcal epidemics.

Bovine mastitis due to group A streptococci occurs, although rarely, in nature and can be produced with ease experimentally by introducing a few organisms into the cistern of the cow's udder by means of a catheter (2). The question naturally arises in this connection how group A streptococci, which are unable

to proliferate in milk after it leaves the cow, can, once they gain entrance to the cow's udder, establish an infection therein without being destroyed by the lactenin of the milk. In view of our demonstration that lactenin is inactive under conditions of reduced oxygen tension, it appeared possible that the milk in the cow's udder might be in a reduced state, and could not, therefore, suppress the growth of lactenin-sensitive organisms.

To investigate this possibility, it was desirable to know the oxidation-reduction potential of milk within the cow's udder. It was not possible to make direct potential measurements within the udder. However, Jackson showed in 1936 (3) that milk withdrawn from the udder anaerobically promptly reduced methylene blue, and he concluded that milk in the udder must also be in the reduced state. We have confirmed Jackson's observations and have investigated the state of activity of lactenin in anaerobically withdrawn milk, as reported in the following experiments:—

Two series of 25 × 150 mm. tubes were prepared, each tube containing 1.0 ml. of 1:10,000 aqueous methylene blue solution, and the tubes of one series containing, in addition, a 2 inch layer of mineral oil. All tubes were sterilized at 121°C. for 20 minutes. Milk was withdrawn from the cow's udder by means of a No. 4 French rubber catheter.¹ After washing out the catheter by allowing milk to flow through it for a few moments, the free end of the catheter was plunged below the surface of the mineral oil, and approximately 10 ml. of milk was allowed to run into the tube and to mix with the methylene blue solution. Similar amounts of milk were drawn into tubes containing no oil, to serve as aerobic controls. Sterile precautions were used throughout.

The milk which lay under the oil decolorized the methylene blue within a few minutes. On standing, it became oxidized very slowly but even after 24 hours incubation at 37°C. only a thin layer of oxidized dye was visible below the fat layer of the milk. Much to our surprise, milk collected and kept without an overlying layer of oil also reduced methylene blue, although in this case color returned to the dye within approximately 30 minutes due to oxidation of the milk by the air.

Inocula of 10⁻⁵ ml. of blood broth cultures of strains 327W (group A) and 090R (group B) were introduced into the milk layer of tubes which contained mineral oil and those which did not, and into a similar pair of tubes which had been immersed in a boiling water bath for 15 minutes to inactivate the lactenin. After 24 hours incubation at 37°C., colony counts were made of material from each of the tubes.

The results, given in Table I, show that milk collected anaerobically and maintained under oil was unable to inhibit growth of a lactenin-sensitive group A streptococcus, whereas milk collected aerobically and incubated with free contact with the air contained active lactenin. It seems reasonable to conclude

¹ The authors are indebted to Dr. E. F. Waller of the Agricultural Experimental Station of the University of Delaware, Newark, for cooperation in obtaining these specimens.

that the intramammary milk is in a reduced state and that lactenin, as long as it is in that site, is inactive and cannot serve as a barrier to the establishment of infection by any group A streptococci which reach it. Upon exposure to the air on milking, lactenin is activated and thereafter can suppress the growth of sensitive streptococci which become implanted in the milk.

Attempts to Confer Protection in Vivo with Lactenin.—The presence of an antistreptococcal substance in the natural food of nurslings prompted an investigation of a possible protective action by the substance. Since milk is taken in large quantity and at frequent intervals by nursing mammals, it appeared reasonable that even a weakly protective action might be of significance, provided the

TABLE I
Inactivity of Lactenin in Milk Drawn from the Udder and Maintained without Contact with the Air

Strains	Colony counts			
	Fresh milk drawn and maintained under oil	Fresh milk free contact with air	Boiled milk drawn and maintained under oil	Boiled milk free contact with air
327W (group A)	67,000,000	31	89,000,000	74,000,000
090R (group B)	430,000,000	270,000,000	360,000,000	310,000,000

material failed to be destroyed by digestive enzymes, was absorbed, and was active in the tissues and fluids of the host.

To demonstrate such an *in vivo* protective action a group of mice was placed on a diet consisting exclusively of fresh cow milk in an attempt to gain the largest possible milk intake. Another group of mice was placed on a boiled milk diet and a third group was kept on a standard pellets and water regime. After several days on these diets, the mice were given intraperitoneal inoculations of strain D58/44/2, a highly virulent mouse-adapted group A streptococcus (which regularly killed in a dilution of 10^{-8} cc., containing 1 to 3 organisms).

No protective action by the fresh milk was observed (Table II).

Since it was possible that failure to obtain protection in the preceding experiment was a result of lack of contact of lactenin and bacteria, another experiment was performed in which the streptococci and lactenin were mixed and immediately thereafter the mixture was injected into the peritoneum. Again no protective effect was manifest.

To determine whether streptococci which were in the process of being killed by lactenin would retain their virulence, an experiment was performed in which a small number of streptococci was seeded into fresh milk, the mixture then was incubated at 37°C. and at hourly intervals for 10 hours mice were injected with

the culture. Colony counts done simultaneously with the injections showed the streptococci to be dying at an appreciable rate due to lactenin action (Table III). The first survivor appeared after the culture had been incubated 3 hours and the colony count was 485 per ml. With continued incubation the colony

TABLE II

Effect of Fresh and Boiled Milk Diets on Susceptibility of Mice to Streptococcal Peritonitis

Mice were kept on indicated diets for 3 days prior to performing virulence tests. Strain D58/44/2 (group A, type 3), was grown 16 hours at 37°C. in neopeptone blood-broth and diluted serially in sterile boiled milk for inoculation. Mice received indicated amount of culture suspended in 0.1 cc. boiled milk intraperitoneally. Colony counts of inoculum showed 108,000,000 colonies per cc. D = death of mouse on indicated day after inoculation. S = surviving at 14 days.

Inoculum	Mice fed fresh milk	Mice fed boiled milk	Mice fed pellets and water
cc.			
10 ⁻⁵	D1 D1 D1 D1	D1 D1 D1 S	D1 D1 D1 D1
10 ⁻⁶	D1 D2 D2 D2	D1 D2 D2 D3	D1 D1 D1 D5
10 ⁻⁷	D1 D1 D1 D1	D1 D4 S S	D1 D1 D1 S
10 ⁻⁸	D2 D2 D3 S	D1 D2 D2 D3	D1 D1 D3 S

TABLE III

Effect on Virulence of Incubating Group A Streptococcus in Fresh Milk

Strain D58/44/2 (Group A, type 3) was grown in neopeptone blood-broth for 16 hours at 37°C. 10⁻⁸ ml. inoculated into 50 ml. sterile fresh cow milk and incubated at 37°C. At hourly intervals 0.1 ml. specimens withdrawn for colony counts and intraperitoneal injections into mice. Symbols as in Table II.

Hrs. of incubation	Colony counts	Fate of mice				
1	705	D1	D1	D1	D1	D1
2	485	D1	D1	D1	D1	D1
3	485	D1	D1	D1	D1	S
4	340	D1	D1	D1	S	S
5	160	D1	S	S	S	S
6	126	D1	D1	D2	D2	S
7	78	D2	S	S	S	S
8	42	D2	D2	D2	D4	S
9	27	S	S	S	S	S
10	9	S	S	S	S	S

counts fell and the number of survivors irregularly increased, so that all 6 mice who had received 9 hour cultures survived, although 27 colonies grew out on blood agar. Numerous virulence studies with this strain of streptococcus grown in broth indicate that most mice receiving that number of organisms from a blood-broth culture will die within a few days. The lactenin,

therefore, had injured the streptococci to the point that the mice could effectively dispose of them, but not to the point that they were unable to grow when planted in blood agar.

In view of the important effect the oxidation-reduction potential has on lactenin activity as shown in the preceding paper (4), the possibility arose that the potential of the peritoneal cavity might be too low for lactenin to be effective, even though it should reach that site unharmed after ingestion. Fildes (5) has demonstrated that the E_h of the peritoneal cavity, as measured by indicator dyes, is sufficiently low to reduce methylene blue, but that in the subcutaneous tissues the potential is considerably higher.

TABLE IV

Effect of Feeding Fresh and Boiled Milk on Course of Subcutaneous Streptococcal Infection in Mice

Mice were placed on diets of the type indicated for 3 days, then injected subcutaneously in the shaved abdomen with the indicated dose of 16 hour blood-broth culture of D58/44/2 diluted in boiled milk. Diets were continued through period of observation. Symbols as in Table II.

Dosage	Fresh milk diet	Boiled milk diet	Pellets and water
<i>ml.</i>			
10 ⁻²	D2 D3 D3 D1	D2 D3 D2 D1	D5 D6 S D6
10 ⁻³	D3 D2 D3 D3	D2 D3 D4 D5	D5 D6 D7 D12
10 ⁻⁴	D2 D4 D7 D10	D1 D2 D4 S	D5 S D5 S
10 ⁻⁵	D3 D2 D2 D4	D5 D2 S D7	S S D5 D10
10 ⁻⁶	D3 D2 S D7	D4 S S S	D5 S D6 D7
10 ⁻⁷	D5 S D5 D5	D2 D6 D3 S	S S S D13

Attempts were made to protect mice from streptococcal inocula given subcutaneously. A few comments are in order concerning the subcutaneous infection of mice by group A streptococci, since infection by that route of inoculation is not commonly employed in streptococcal studies.

When strain D58/44/2, which as shown in Table II is highly virulent by the intraperitoneal route, was diluted in boiled milk and administered subcutaneously into mice fed a standard diet of pellets and water, death rarely occurred before the 5th day, no matter how large the inoculum, and some mice recovered after receiving as much as 10⁻² ml. Local lesions developed even with small doses (10⁻⁷ ml.) but ultimate recovery was the rule. Boiled milk was used as the diluent for the culture for inoculation because lesions were produced with fewer organisms than when the culture was diluted in broth. The subcutaneous infection appeared to offer an opportunity to determine whether lactenin would confer protection in the face of a relatively slowly developing infection in a site where the E_h of the tissues was higher than in the peritoneal cavity.

Table IV shows that mice fed milk, either fresh or boiled, died more rapidly and with smaller doses of streptococci than did mice fed pellets and water. No

attempt will be made to explain the detrimental effect of milk diets, other than to point out that milk is an abnormal diet for weaned mice; but it is certain that the ingested lactenin was inadequate to overcome the infection, either because it was destroyed in the intestinal tract, because it did not achieve a sufficiently high concentration at the site of infection, or because it was inactivated there, possibly by the low oxidation-reduction potential of the tissues.

DISCUSSION

Although milk-borne streptococcal epidemics are dramatic and attract considerable attention when they occur, they actually are rather rare events. For example, between 1923 and 1947 only 202 such epidemics, averaging considerably less than 10 per year, were reported by state and local health authorities in the United States (6). During this same period more than twice as many outbreaks of milk-borne typhoid fever were reported. Considering the widespread use of milk under a variety of commercial and domestic conditions and the high incidence of streptococcal carriers capable of contaminating the milk, it would be surprising not to encounter streptococcal epidemics of this type more frequently if fresh milk were, in fact, a good medium for the growth of group A streptococci.

Milk-borne streptococcal outbreaks are almost always related to the consumption of unpasteurized milk. For example, of 57 epidemics investigated by the New York State Health Department between 1917 and 1941 all but one were traced to the use of raw milk, and the exception followed the use of milk labeled "pasteurized" although evidence indicated that it had not been properly processed (7).

By means of serological technics, which make it possible to trace the source of the infecting streptococcus, it has been established beyond reasonable doubt that most milk-borne epidemics originate from mastitis in the cow due to group A streptococcus, rather than to contamination of the milk after milking. In a number of epidemics, which have been reviewed by Ernst (8) and need not be recapitulated here, a strain belonging to the same serological type of group A streptococcus was recovered from patients in the epidemic, from the udder of a cow supplying milk, and from a dairyman who had had a streptococcal infection prior to the onset of the mastitis and had had contact with the cow.

The numbers of group A streptococci discharged in the infected milk are variable, but as many as 820,000 per ml. were recovered by Bendixen and Minett from a carefully studied cow with naturally acquired chronic infection (9). When such large numbers are excreted it is not necessary to postulate multiplication in the milk after milking to account for the epidemic. The rapidity with which these streptococci will die after the milk has become oxidized and the lactenin activated, will depend on the temperature at which the milk is stored. But even at the optimal temperature for lactenin action it

takes many hours for the milk to become entirely free of lactenin-sensitive streptococci.

The typical milk-borne streptococcal epidemic is seen, therefore, to be a rare event which occurs when a cow acquires group A streptococcal mastitis from contact with a human carrier, when streptococci are discharged in the milk in large numbers, and when the milk is distributed unpasteurized to the consumer. Lactenin fails to prevent mastitis because it is inactive in the udder due to low oxidation-reduction potentials in that site. After milking, the lactenin becomes active and serves to repress multiplication of lactenin-sensitive streptococci which may become implanted in the milk.

There are two classes of exceptions to the typical milk-borne epidemic just described. In the first massive direct contamination of drawn milk occurs so that, without proliferation, sufficient streptococci are present in the milk to survive until the milk is consumed. An example of this is the milk-borne epidemic at Vejle, Denmark, (8) in which streptococcal pus from a profusely discharging otitis media dropped into the milk. The other class of exceptions involves the use of preserved milk preparations, such as canned and powdered milks, in which the lactenin has been destroyed in the process of preservation. Such preparations are excellent media for the growth of group A streptococci. Canned and powdered milk are usually diluted or reconstituted in the home. Since the mother or whoever prepares the milk has abundant opportunity to transmit infection to the other members of the household by other routes, suspicion rarely falls on milk as a vehicle of transmission. In institutions, however, in which preserved milk may be prepared some time before its consumption by a population having little or no direct contact with the cooks, epidemics resulting from proliferation of streptococci in the lactenin-free milk are readily recognized. Three epidemics of this type were reported from military institutions in World War II (10-12). Two of these involved powdered milk and in the other canned milk was used. The milks were prepared with warm water several hours before consumption. In two of the epidemics streptococci of the same serological type were recovered from patients' throats and from the individual who prepared the milk. Facilities for serological typing were not available in the third epidemic, but hemolytic streptococci were recovered from the patients, from the device which was used to reconstitute the milk, and from the cook who had prepared the milk. Thus there is little doubt that milk in which the lactenin has been destroyed by heat can, on becoming contaminated, serve as a dangerous vehicle for the spread of streptococcal infections.

The studies which have been presented showing that lactenin, administered orally and parenterally, failed to protect mice from group A streptococci injected intraperitoneally or subcutaneously, have little bearing on any protective role lactenin may have under natural conditions. The predominant human infection with the streptococcus is one of the mucous membranes of the upper

respiratory tract. It is conceivable that the nursing infant may ingest sufficient milk to allow a significant concentration of lactenin to reach the mucous membranes where, on contact with the oxygen of the atmosphere, it might become oxidized and exercise an antistreptococcal function. Only a direct study of this matter in the nursing infant can illuminate the problem. At present there is no positive evidence that lactenin under natural circumstances is capable of combating infection.

On the basis of studies by earlier workers and of the investigations presented here, the chief characteristics of lactenin may be summarized as follows: Lactenin is bactericidal for certain hemolytic streptococci, particularly those of group A. It has a temporary bacteriostatic effect on others and is without any perceptible effect on the remainder. Lactenin is a large molecule or is bound to a large molecule, since it is held back by dialyzing membranes. It is heat-labile, being inactivated by 80° C. or higher, but not being destroyed by commercial pasteurization of milk. It is not digested by trypsin or chymotrypsin. It is inactive when oxygen is removed or when sulfhydryl reducing agents are added. It is inactivated by large amounts of thiamine, but not by excesses of other growth factors. It is not identical with any of the known antibacterial enzymes of milk (xanthine oxidase, lysozyme, peroxidase). The lactenin of goat and human milk inhibits the same streptococcal strains that are inhibited by cow milk lactenin. Lactenin is not present in the serum of cows whose milk contains it, so that apparently it is formed by mammary tissue. It is not an actively induced antibody; it is not complement and does not require complement for its action. Whether it ever acts under natural circumstances to prevent infection is questionable. The inability of lactenin to prevent establishment of group A streptococci in the cow's udder is explained by the low oxidation-reduction potential in the udder, which renders lactenin inactive. Lactenin is activated when milk is exposed to the air on milking, and thereafter serves to inhibit the growth of lactenin-sensitive organisms which may become implanted in the milk, which accounts for the infrequency of milk-borne epidemics due to direct contamination of the milk after milking. Canned and powdered milk, in which the lactenin has been destroyed by heat, constitute excellent media for the growth of group A streptococci and epidemics due to direct contamination of such milk preparations have been reported.

SUMMARY

The ability of lactenin to prevent the multiplication of group A streptococci when milk becomes contaminated with that organism accounts in part at least, for the infrequency of milk-borne streptococcal epidemics. From epidemiological studies it has been shown that most such epidemics arise from the consumption of raw milk in which streptococci occur as a result of bovine mastitis due to group A streptococcus. Lactenin fails to prevent the establishment of

mastitis due to the group A streptococcus because the milk in the cow's udder is at a low oxidation-reduction potential and the lactenin is inactive.

Lactenin, being destroyed by temperatures of 80°C. or above, is absent from canned and powdered milk. When the latter have been diluted or reconstituted, they can serve as excellent growth media for group A streptococci, and epidemics have occurred as a result of contamination of milk supplies of those types.

The administration of lactenin by mouth or intraperitoneal injection failed to protect mice from peritonitis or subcutaneous infection due to group A streptococcus.

BIBLIOGRAPHY

1. Pullinger, E. F., and Kemp, A. E., *J. Hyg.*, Cambridge, Eng., 1937, **37**, 527.
2. Little, R. B., and Plastridge, W. N., *Bovine Mastitis*, New York, McGraw-Hill Book Co., 1946
3. Jackson, C. J., *J. Dairy Research*, 1936, **7**, 31.
4. Wilson, A. T., and Rosenblum, H., *J. Exp. Med.*, 1952, **95**, 39.
5. Fildes, P., *Brit. J. Exp. Path.*, 1929, **10**, 197.
6. Summary of Milk-Borne Disease Outbreaks Reported by State and Local Health Authorities during the Years 1923-1947, Washington, D. C., Federal Security Agency, 1949.
7. Dublin, T. D., Rogers, E. F. H., Perkins, J. E., and Graves, F. W., *Am. J. Pub. Health*, 1943, **33**, 157.
8. Ernst, J., *The Epidemiological Significance of Grouping and Typing the Hemolytic Streptococci*, Copenhagen, E. Munksgaard, 1942.
9. Bendixen, H. C., and Minett, F. C., *J. Hyg.*, Cambridge, Eng., 1938, **38**, 374.
10. Allen, R. F., and Baer, L. S., *J. Am. Med. Assn.*, 1944, **124**, 1191.
11. Wilson, T. S., *Brit. Med. J.*, 1944, **2**, 275.
12. Purvis, J. D., and Morris, G. C., *U.S. Naval Med. Bull.*, 1946, **46**, 613.