THE ANTISTREPTOCOCCAL PROPERTY OF MILK

I. Some Characteristics of the Activity of Lactenin in Vitro. The Effect of Lactenin on Hemolytic Streptococci of the Several Serological Groups

By ARMINE T. WILSON, M.D., and HERMAN ROSENBLUM, M.D.

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

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The ability of cow milk to inhibit the growth of several species of bacterium was first described by Hesse in 1894 (1), and has been studied periodically since that time. The basic observation in relation to the streptococcus was that when certain strains of that organism were inoculated into fresh cow milk they died. Other streptococcal strains grew well. When the milk was boiled all strains multiplied in it without hindrance. It was assumed that the milk contained a heat-labile antistreptococcal substance, and this substance was named "lactenin" by Jones and his collaborators, whose work has been outstanding in this field.

The properties and actions of lactenin as currently known may be summarized as follows: Lactenin is present in the whey fraction of milk, will not pass through a dialyzing membrane, and is inactivated by exposure to a temperature of 80°C. or higher but survives pasteurization unharmed. It is not digestible by trypsin and thus can be separated from much inert milk protein by tryptic digestion of milk followed by dialysis. It has not been prepared in pure form and its chemical nature is therefore unknown, but its heat lability and non-dialyzability have suggested to several investigators that it might be an enzyme. It is readily injured by acid and alkali and by many reagents used for protein fractionation, but in its native state in milk it is quite stable, surviving at least several weeks when stored at 6°C. It is readily adsorbed by a number of solid adsorbants, but has not been successfully eluted from them.

Lactenin was found to be present in the milk of all cows, although it varied in titre from cow to cow, from time to time in the milkings of a single cow, and even in milk from the 4 quarters of the same cow at the same time. It was present in low titre in colostrum, reaching a relatively stable level 4 to 5 days postpartum and persisting thereafter in the milk (3). Its presence in milk appeared to be due to formation in the mammary gland itself, rather than by excretion through the gland, since it was not present in the blood of cows whose milk contained it (2). It has not been studied in the milk of species other than the cow.

Jones and Simms found that two strains of streptococcus differed in their susceptibility to lactenin (4–7). A non-hemolytic streptococcus obtained from the

udder of a cow with bovine mastitis failed to grow in the presence of lactenin for 5 to 6 hours but thereafter it multiplied rapidly. On the other hand, multiplication of a scarlet fever strain was completely suppressed, and the inoculated organisms ultimately died. Both strains grew well in milk in which the lactenin had been inactivated by boiling. The ultimate ability of the non-hemolytic organism to grow in fresh milk was attributed to the development of a lactenin-resistant variant, since organisms from a fully grown fresh milk culture showed no delay in multiplication on subculture into a new specimen of fresh milk (5).

Jones and Little postulated a protective function for lactenin, assuming that its presence in intramammary milk would inhibit the growth of streptococci which might gain access to the udder (2). Jones found, however, that milk from infected quarters contained active lactenin just as did milk from uninfected quarters (3), so the occurrence of the mastitis could not be attributed to a failure of the involved quarters to form it.

Lactenin was not utilized or destroyed in the course of its action, and it was not adsorbed from milk by heat-killed suspensions of sensitive bacteria. Immunization of cows with lactenin-sensitive streptococci did not cause an increase in the lactenin output of the milk (2). Lactenin therefore, did not appear to be an antibody. It was not similar to serum complement and did not depend on complement for its action.

The purpose of the present investigation was to study the factors which influence the activity of lactenin *in vitro*, and consequently its measurement in the laboratory, to study the action of lactenin on streptococci of the several serological groups and types, to study the effect of chemical and physical agents on lactenin in the hope that some insight would be gained into its nature and mode of action, to determine whether or not it might be of any protective value against infection *in vivo*, and to consider its role in milk-borne streptococcal epidemics.

Methods and Materials

Milk.—The cow milk used in these studies was ordinary market milk obtained from a dairy which pooled the milkings from 95 herds of cattle. It was pasteurized at the dairy by the holding process at 61°C. for 30 minutes. Fat was removed in the laboratory by centrifugation. Human milk was obtained from the Human Milk Bank of the Delaware Hospital, Wilmington, through the courtesy of Dr. Margaret Handy. It had not been pasteurized or otherwise sterilized. Goat milk was obtained from a commercial farm and had not been pasteurized.

Sterilization of Milk.—Heat could not be used for this purpose, since lactenin was inactivated thereby. Passage of either milk or whey through bacterial filters was a laborious and unsatisfactory procedure, since the filters rapidly became plugged and only the coarsest filters allowed lactenin to pass. It was found that large quantities of milk or whey could readily be sterilized by liquid ethylene oxide in a final concentration of 0.5 per cent, and most of the experiments reported here were performed with milk sterilized in that manner. A detailed description of the sterilizing procedure has been presented elsewhere (9). Ethylene oxide did not inactivate lactenin and the growth-supporting qualities of milk were unimpaired by the treatment.

Demonstration of Lactenin Activity of Milk.—The presence of lactenin could be demon-

strated most easily by inoculating tubes of fresh sterile milk (usually 2 ml. per tube) with a small inoculum (usually 10^{-7} ml. of a 16 hour blood broth culture) of test organism. Two strains of streptococcus were used for testing, one known to be highly sensitive and the other known to be highly resistant to lactenin. The inoculated tubes were incubated overnight at 37°C., and a loopful from each was then plated on blood agar. Proliferation of the sensitive strain was shown to have been inhibited because few or no colonies developed on the plate, whereas growth of the resistant strain was shown to have proceeded unchecked, innumerable colonies developing on the plate from a loopful. For purposes of control, tubes containing milk which had been boiled to inactivate the lactenin were inoculated with the two strains, incubated, and subcultured for growth. The lactenin-sensitive as well as the lactenin-resistant strain grew well in the boiled milk. For more detailed studies of lactenin activity, colony counts were made of the fresh and boiled milk cultures at suitable intervals by standard serial dilution procedures. A similar method was used to determine the lactenin sensitivity of various streptococci using milk known to contain lactenin.

Jones and Simms (6) measured lactenin activity by pouring peptone-blood agar plates to which fresh or boiled milk and a small streptococcal inoculum had been added before pouring. The diameters of the hemolytic zones which surrounded colonies developing on boiled and fresh milk agar plates were compared, the difference in magnitude constituting an index of lactenin activity. Later in this paper the factors which affect the results of this test will be considered.

Titration of the lactenin content of milk specimens was accomplished by diluting fresh, sterile milk with boiled milk in multiples of two, inoculating with a lactenin-sensitive streptococcus, incubating overnight, and subculturing the series of tubes on blood agar plates. A tube containing only boiled milk similarly inoculated served as control. The lactenin titre of the milk was that dilution of fresh milk in total mixture which completely inhibited growth of the test streptococcus, and in the case of cow milk, usually amounted to 1:2 to 1:16.

The most satisfactory method for testing the lactenin sensitivity of streptococcal strains on a large scale involved the use of a solid medium made by mixing equal volumes of fresh sterile milk and 3.6 per cent sterile agar-agar at 45°C. and pouring plates. Similarly prepared plates employing boiled milk served as lactenin-free controls. Susceptible strains failed to grow or at best produced pinpoint colonies on the fresh milk agar plates, whereas resistant strains grew well. Both strains grew well on the boiled milk agar plates.

Strains.—Two classes of streptococcal cultures were used in this study. The first consisted of stock strains of the several streptococcal groups and of the type strains within group A. These were obtained from the collection of Dr. Rebecca C. Lancefield of the Rockefeller Institute Hospital to whose generosity we are greatly indebted. A few group B strains of bovine origin were obtained from Dr. J. C. Kakavas of the University of Delaware. In addition, certain lyophilized strains, chiefly belonging to group A, originated from the Streptococcal Laboratory of the Naval Medical Center, Bethesda.

The second class consisted of fresh strains obtained from patients with acute streptococcal infections or from streptococcal carriers, and were tested for lactenin susceptibility within a few days of their primary isolation. Such strains were obtained from patients in the Alfred I. du Pont Institute, from The Temple University Hospital, Philadelphia, through the courtesy of Dr. Theodore Anderson, and from Fort Francis E. Warren, Wyoming, where an active streptococcal epidemic was in progress.[1] Another group of strains, which had been recovered from patients and carriers in a normal population study, was obtained from Cleve-

[1] The senior author is indebted to the Commission on Streptococcal Diseases of the Armed Forces Epidemiology Board for making possible the study at Fort Warren; to Col. John K. Cullen and to Dr. Charles Rammelkamp and his associates for generously making available the facilities of the streptococcal laboratory at Fort Warren.

land, through the courtesy of Dr. George Feller, of the Department of Preventive Medicine, Western Reserve University.

All the strains in this study were identified serologically by the capillary precipitin method (10) with sera supplied by the Communicable Disease Center, Public Health Service, Chamblee, and by Dr. Rebecca Lancefield.

EXPERIMENTAL

Effect of Size of Inoculum on Lactenin Activity.—

Fresh milk which had been sterilized with ethylene oxide was distributed in a series of small test tubes, and increasing amounts of a blood-broth culture of streptococcus 327W (a strain belonging to group A, type 1, originally isolated from a milk-borne streptococcal epidemic) were introduced into the several tubes. The inocula were of such size that the tubes received from 1,290 to 12,100,000 organisms per ml. of milk at the beginning of incubation. Another series of tubes containing milk which had been boiled to inactivate the lactenin was similarly inoculated, and served as control.

It can be seen (Table I) that no matter how small the initial inoculum was, bacterial proliferation occurred at least during the first 4 hours. With small inocula (12,900 organisms or fewer per ml. of milk) the lactenin-containing milk sterilized itself within 24 hours. With inocula of intermediate size the numbers of streptococci decreased although the culture did not become sterile within that time. With the largest inoculum (12,100,000 organisms per ml.) a distinct repression of growth was in evidence although the number of bacteria present at the end of 24 hours was greater than that introduced by inoculation.

When solid milk-agar plates were used (see Methods) and the streptococcal inoculum was streaked on the surface of the plates, even a large inoculum (such as a loopful, consisting of approximately 10^{-3} ml. of blood broth culture) was effectively inhibited by the fresh milk. Occasionally pinpoint colonies of lactenin-sensitive strain developed on fresh milk agar, but usually no growth whatever could be seen, and in any case the difference in growth on fresh and boiled milk agar was great enough to be readily recognized. Varying the inoculum size had little if any effect on the results of testing by this method.

Effect of Type of Medium on Lactenin Activity.—In the preceding experiments the milk served not only as the source of lactenin but also as the medium for the growth of the test streptococci. When normal human, rabbit, sheep, or horse serum was added to the milk in a final concentration of 5 per cent, some improvement in growth occurred and no inactivation of lactenin was observed. When defibrinated whole blood (rabbit, sheep, or human) was incorporated into the milk-agar plates in a final concentration of 5 per cent, a definite, although not complete, inactivation of lactenin occurred, pinpoint or somewhat larger colonies being produced by inoculation of a strain of streptococcus which was fully inhibited by plain milk-agar.

The incorporation of peptone in a final concentration of 1 per cent in the milk-agar plates produced a very marked inactivation of lactenin as tested

TABLE I

Effect of Inoculum Size on Lactenin Action

Skimmed cow milk was sterilized with ethylene oxide. A portion of the sterilized milk was then immersed in a boiling water bath for 5 minutes to inactivate lactenin. 4.9 ml. portions of fresh and boiled milk were placed in series of tubes and inoculated with 0.1 ml. of the indicated dilutions of 16 hour blood-broth culture of strain 327W. At indicated times tubes of fresh and boiled milk were withdrawn and colony counts were made to estimate bacterial growth.

Inoculum	Growth period	Colony counts	
		Fresh milk	Boiled milk
ml.	*hrs.*		
10^{-5}	0	(1,290)*	(1,290)*
(6,450 organisms)	4	2,303	33,060
	8	485	2,860,000
	16	0	63,000,000
	24	0	24,500,000
10^{-4}	0	(12,900)	(12,900)
(64,500 organisms)	4	20,950	950,000
	8	8,800	17,760,000
	16	50	25,000,000
	24	0	37,500,000
10^{-3}	0	(143,000)	(143,000)
(715,000 organisms)	4	705,000	15,000,000
	8	343,000	87,000,000
	16	170,000	88,500,000
	24	4,000	107,000,000
10^{-2}	0	(1,040,000)	(1,040,000)
(5,200,000 organisms)	4	5,300,000	146,000,000
	8	3,610,000	230,000,000
	16	3,750,000	523,000,000
	24	24,000	409,000,000
10^{-1}	0	(12,100,000)	(12,100,000)
(66,500,000 organisms)	4	109,000,000	457,000,000
	8	176,000,000	1,310,000,000
	16	185,000,000	1,790,000,000
	24	153,000,000	1,480,000,000

* The number of streptococci per milliliter of milk at the beginning of growth was calculated from colony counts of the inoculum added and is shown in parentheses.

by streaking a loopful of whole blood broth culture on the surface of the plate. This finding was surprising in view of Jones and Simms' method for measuring lactenin activity, in which a small inoculum of streptococcus was

added to milk-peptone-blood agar before plates were poured (6). Investigations of the apparent discrepancy in observation revealed that some peptones inhibited lactenin when the inoculum was large, but when the inoculum was small (10^{-7} ml. of culture, containing approximately 50 organisms) lactenin was active. This was true whether the inoculum was incorporated into the medium before pouring the plate or was streaked onto the surface of a poured plate.

Several peptones and related preparations were studied for ability to inhibit lactenin action. Most of these were commercial articles, but some were prepared in the laboratory. Some of the peptones inhibited lactenin no matter how small the inoculum or how large the quantity of milk used. Others inhibited lactenin only when a large inoculum was employed. Of the commercial peptones, bacto-tryptose (Difco) was found to be as satisfactory as any for use in a medium for studying lactenin. A medium consisting of 2 per cent tryptose, 0.5 per cent NaCl, 2 per cent agar, 4 per cent defibrinated blood, and 15 per cent milk was suitable for demonstrating the susceptibility of a streptococcus given in inoculum of not more than 50 colonies per plate. The same medium could be used for measuring the lactenin activity of milk when a known sensitive strain was used for testing.

The quantitative method of Jones and Simms is subject to one drawback which has rendered it inapplicable in many instances in which we wished to measure lactenin. It depends on measurement of the zone of hemolysis produced by the growing streptococcus, and the size of this zone is influenced by the amount of lactose present in the medium or by the effect of other substances which it may be desired to add as well as by inhibition of multiplication of the organism by lactenin. Thus, when milk or whey is diluted, not only the diminishing amount of lactenin but also the diminishing amount of lactose affect the size of the hemolytic zone. Certain lactenin-containing preparations and certain substances which we wished to add to milk to determine their effect on lactenin cause blood to become brown, and this color change was associated with a decreased susceptibility of the erythrocytes of the culture plate to lysis, although growth of the streptococcus, as judged by size of the colony itself, was not adversely effected. The assay method of Jones and Simms must be limited, therefore, to circumstances in which ingredients of the medium have little or no direct effect on hemolysis; and, in any case, it cannot be considered an absolute measurement of growth inhibition by lactenin.

Effect of Temperature on Lactenin Activity.—Lactenin itself is irreversibly inactivated by heat, the minimum temperature required being about 80° C. For practical purposes in the laboratory, inactivation is accomplished by immersing tubes or flasks of milk in a boiling water bath for 5 to 15 minutes.

Below the point of inactivation, temperature affects the rapidity and completeness with which lactenin acts. However, measurement of the influence of

temperature on lactenin is complicated by the direct effect of temperature on the rate of bacterial multiplication. Thus, the number of living streptococci present in a fresh milk culture after incubation at a particular temperature is the resultant of the ability of the organism to proliferate at that temperature and of the ability of lactenin to inhibit proliferation at the same temperature. Table II shows that a few streptococci inoculated into fresh and boiled milk, and incubated at 4°C., failed to multiply but were not killed. The organism used was known to be sensitive to lactenin at higher temperatures, but no action of lactenin on it at that temperature was shown, since the numbers of living bacteria remained the same in both fresh and boiled milk. The stationary growth status of the culture must, therefore, be attributed to the inhibitory

TABLE II

Effect of Temperature on Lactenin Activity

To 4.9 ml. of milk brought to the temperature at which it was to be incubated, was added a small inoculum of streptococcus 327W. Colony counts were made of the original inoculum and of the milk cultures after incubation for 18 to 26 hours.

Temperature of incubation	Type of milk	Size of inoculum (Colonies per ml. milk)	Colony counts at 18 to 26 hrs.
°C.			
4	Fresh milk	840	810
	Boiled milk	840	850
22	Fresh milk	1,540	990
	Boiled milk	1,540	9,000,000
37	Fresh milk	510	0
	Boiled milk	510	9,150,000

effect of temperature alone on proliferation of the organism, and no additional bacteriostatic or bactericidal effect of lactenin need be considered. When similarly inoculated tubes of fresh and boiled milk were incubated at 22°C., a slight decline in numbers of living organisms took place in the fresh milk, whereas free proliferation occurred in the boiled milk; thus, a definite, although not maximal, lactenin effect occurred at 22°C. Incubation at 37°C. lead to sterilization of the fresh milk culture which represents maximal lactenin action, and that temperature also favored maximal proliferation in the boiled milk culture.

Effect of Cow Milk on Streptococci of the Several Serological Groups and Types.—No work, to the best of our knowledge, has been done on lactenin since the development of serological methods for the differentiation of streptococci. In earlier work the organisms studied were classified by source and by biochemical reactions. It will be remembered that Jones and Simms (5) studied

two streptococcal strains, one a hemolytic organism from scarlet fever (probably a group A streptococcus) which was highly sensitive to lactenin, the other a non-hemolytic streptococcus from classical bovine mastitis (probably a group B or C strain) which, after a preliminary period of inhibition, proliferated freely in fresh milk. Pullinger and Kemp (8), worked with three strains which were not identified serologically, but presumably were members of group A.

It appeared desirable, therefore, to study the effects of lactenin on numerous streptococcal strains which had been assigned to the proper serological group and type, and to determine whether such sensitivity was associated with

TABLE III

Growth of Streptococci of Several Serological Groups in Fresh and Boiled Milk

50 ml. of sterile fresh and boiled cow milk were inoculated with 10^{-6} ml. of an 18 hour broth culture of the indicated strain and incubated at 37°C. Portions were removed at 4, 6, and 24 hours for performing colony counts.

Group	Strain	Colony counts							
		Fresh milk				Boiled milk			
		0 hrs.*	4 hrs.	6 hrs.	24 hrs.	0 hrs.*	4 hrs.	6 hrs.	24 hrs.
A	327W	(1,740)	2,130	600	1	(1,740)	353,000	1,520,000	19,000,000
B	090R	(2,980)	241,000	2,470,000	470,000,000	(2,980)	600,000	10,800,000	256,000,000
B	K4	(466)	18,100	2,610,000	116,000,000	(466)	104,500	1,950,000	154,000,000
B	K2	(1,600)	3,670	5,100	52,000,000	(1,600)	13,750	73,000	800,000,000
C	H69	(1,480)	64,000	530,000	470,000,000	(1,480)	2,860,000	54,000,000	230,000,000
C	C496	(6,018)	250,000	3,520,000	2,920,000,000	(6,018)	380,000	3,000,000	1,020,000,000
D	D178A	(2,780)	310,000	9,300,000	340,000,000	(2,780)	1,620,000	66,000,000	3,200,000,000
D	D178B	(4,660)	600,000	2,350,000	180,000,000	(4,660)	600,000	3,000,000	1,440,000,000
L	K130	(3,260)	2,780	3,050	4,000,000	(3,260)	1,650,000	49,500,000	1,190,000,000
L	D167C	(3,680)	376,000	14,400,000	1,260,000,000	(3,680)	350,000	6,700,000	2,770,000,000

* The number of streptococci per milliliter of milk at the beginning of growth was calculated from colony counts of the inoculum added, and is shown in parentheses.

particular groups and types, or whether it was distributed at random among them.

Two types of investigation into this matter were made. Several strains, belonging to groups A, B, C, D, and L, were seeded into fresh and boiled milk and colony counts were made at 4, 6, and 24 hours. In the other study a larger number of strains was tested for lactenin sensitivity by streaking on milk-agar plates and observing growth or inhibition of growth after overnight incubation.

The results of the first part of the study, the technical details of which are given in the table, are shown in Table III. It is seen that all the strains (except K130) had multiplied to some extent by 4 hours. After that time the numbers of viable group A organisms recoverable decreased, until by 24 hours the culture was almost sterile. In the case of the other organisms,

three (C496, D178B, and D167C) showed no apparent effect from the lactenin in the fresh milk, whereas the others showed varying degrees of temporary inhibition of growth, after which proliferation proceeded unchecked. In some cases the final colony counts in the fresh milk were still considerably less than in the boiled milk (strains K2, D178A) whereas in others the converse was true. It thus appeared that the activity of lactenin on various streptococci varied in effect from complete destruction of viable cells in the culture through temporary inhibition of growth to no effect whatever. When the final counts at 24 hours were examined, a very sharp difference was seen between the group A strain, which had largely died off, and the other strains which had multiplied many times, even though final growth was not always as good in the fresh as in the boiled milk. Many strains belonging to the various serological types within group A have been tested for lactenin sensitivity, and although minor strain differences have been encountered, almost all strains have been killed within 24 hours, the remainder becoming sterile within 48 hours.

Advantage has been taken of the striking difference in the amount of growth which various streptococci attain in fresh milk in 24 hours to divide them into two general classes: those which fail to proliferate or have been killed by the end of 24 hours and those which have grown well. The two classes are referred to as being "lactenin-sensitive" and "lactenin-resistant." Such designations are not meant to imply that, in the first case no proliferation can occur at any time in the presence of lactenin or in the second that no lactenin effect is detectable at any time, but simply refer to the end-result of lactenin influence after the lapse of 24 hours.

For the second part of the study, the milk-agar plate method was used, because it lends itself conveniently to the determination of lactenin sensitivity of numerous strains without the prohibitive labor involved in doing colony counts. Each strain was streaked onto segments of three agar plates. One plate contained 50 per cent fresh milk, another contained 50 per cent boiled milk, and the third was a standard peptone-blood agar plate for determination of purity of culture. A preliminary survey, by this method, of strains at hand demonstrated that group A streptococci were inhibited by lactenin, whereas group B and C strains were resistant to it. The study was then extended to a larger group of stock strains, which belonged to all the serological groups except M and N. All the group A stock strains tested were sensitive to lactenin, regardless of serological type. Stock strains of groups B, C, D, and E were all resistant to it. Strains belonging to groups F, G, H, K, and L fell into both classes, some of them being inhibited and others growing well (Table IV).

It was clear from this experience that lactenin sensitivity was not strictly group-associated. Nevertheless, since strains which are cultured from the respiratory tract in acute streptococcal infections belong predominantly to

groups A, C, and G, and strains belonging to the other groups are rarely encountered, it appeared possible that in actual practice testing for lactenin sensitivity might distinguish with reasonable accuracy between the important group A organisms and the less important strains of other groups likely to be encountered under the circumstances. Such a relatively simple biological test

TABLE IV

Lactenin Sensitivity of Streptococci of Various Serological Groups

A 2 mm. loopful of 16 to 24 hr. blood broth culture was streaked on 50 per cent fresh milk agar, 50 per cent boiled milk agar and peptone blood agar. After incubation at 37° C. for 24 hours, the plates were inspected for streptococcal growth. Sensitive strains grew well on boiled milk agar and failed to grow or produced at best pinpoint colonies on fresh milk agar. Resistant strains grew as well or almost as well on fresh milk agar as on boiled milk agar. Intermediate strains grew less well on fresh milk agar than on boiled milk agar, but growth was appreciable.

Group	Total No. of strains	No. sensitive to lactenin	No. resistant to lactenin	No. of intermediate sensitivity to lactenin
Stock strains				
A	69	69	0	0
B	15	0	15	0
C	20	0	20	0
D	19	0	18	1
E	4	0	4	0
F	3	1	1	1
G	8	4	3	1
H	4	0	2	2
K	3	2	0	1
L	10	0	8	2
Freshly isolated strains				
A	408	407	0	1
B	10	1	9	0
C	11	0	10	1
D	6	0	6	0
G	17	1	15	1
?	1	0	1	0

might prove useful in laboratories not equipped to identify streptococci by serological technics. It was therefore decided to give the lactenin-sensitivity test trials under field circumstances with freshly isolated strains. Of 453 such strains which were tested, 408 (90 per cent) belonged to group A, 10 (2 per cent) to group B, 11 (2 per cent) to group C, 6 (1.3 per cent) to group D, 17 (3.7 per cent) to group G, and 1 was ungroupable. All but one of the 408 group A strains were inhibited by lactenin on first testing, and the exceptional strain was found to be fully inhibited on retesting. All of the 45 strains be-

longing to the other groups were resistant to lactenin except 2 strains (B and G) which were sensitive and 2 strains (C and G) which were partially inhibited.

Thus among these freshly isolated strains, all the group A strains were inhibited by lactenin and almost all the strains belonging to groups other than A were resistant to lactenin. Five strains of 453, or slightly more than 1 per cent, departed from the general trend.

Comparison of Lactenin in Human, Cow, and Goat Milk.—It has been shown in the preceding sections that the lactenin of cow milk inhibited the growth of group A streptococci, organisms which usually infect man, but failed to inhibit group B streptococci, organisms which usually infect cows. The question

TABLE V

Comparison of Lactenin in Cow, Human, and Goat Milk

Cow, human, and goat milk specimens were sterilized with ethylene oxide. A sample of each was immersed in boiling water for 15 minutes to inactivate the lactenin. Agar plates were prepared containing 50 per cent fresh or boiled milk. Loopfuls of 18 hour blood broth cultures of the test strains were streaked on surface segments of the plates, which were then incubated at 37° C. for 24 hours, when they were observed for streptococcal growth. ++++ = maximal growth. — = no visible growth.

Strain	Cow milk		Human milk		Goat milk	
	Fresh	Boiled	Fresh	Boiled	Fresh	Boiled
Group A 327W	—	++++	—	++++	—	++++
Group A D205	—	++++	—	++++	—	++++
Group A D58/45/1	—	++++	—	++++	—	++++
Group A J17D	—	++++	—	++++	—	++++
Group B O90R	++++	++++	++++	++++	++++	++++
Group C K106	++++	++++	++++	++++	++++	++++
Group D D76	++++	++++	++++	++++	++++	++++
Group G C133	++++	++++	++++	++++	++++	++++

arose whether milk of other mammals contained lactenin, and, if so, whether it inhibited growth of the same streptococcal groups and strains as did cow milk.

Accordingly human and goat milk were tested by the milk-agar test for ability to inhibit strains of known sensitivity and resistance to cow milk. Table V shows that streptococcal strains which are inhibited by cow milk are also susceptible to human and goat milk, whereas strains resistant to the one are similarly resistant to the others. On this basis, lactenin in human and goat milk appears to be the same qualitatively as that in cow milk.

DISCUSSION

In studying the action of lactenin *in vitro*, the type of medium, size of inoculum and temperature of incubation must be taken into account. When milk alone is used, both as source of lactenin and as growth medium, the size

of inoculum is of minor importance, but when a medium containing peptone is used, the streptococcal inoculum must not be too large, and, indeed, some peptones inhibit lactenin action no matter how small the inoculum. The effect of temperature is important, but not critical in terms of the temperatures usually employed for incubation in the laboratory. The optimal temperature for lactenin activity is about 37°C. When room temperature is reached, its activity is somewhat reduced. When 0°C. is employed, no lactenin effect can be observed, the culture remaining stationary in count owing to temperature alone.

Three general types of medium are suitable for lactenin studies: (*a*) fresh, sterile, liquid milk, (*b*) fresh, sterile milk solidified with agar-agar and (*c*) blood-peptone-agar to which milk is added. In the last case, the streptococcal inoculum must be small, the selection of peptone is important, and when the method employed by Jones and Simms, in which the size of hemolytic zones is used as an index of inhibition of growth, the inclusion in the medium of substances having an effect on hemolysis which is independent of their effect on growth, may result in misleading observations.

The most troublesome technical detail in studying lactenin was to obtain an adequate quantity of sterile milk. The procedure of sterilizing with ethylene oxide appears to be admirably suited to the purpose. A difficulty we encountered in using ethylene oxide which was puzzling until its source was discovered, resulted from failure to allow sufficient time to elapse from the time of adding ethylene oxide until the medium was used. A period of 24 hours is minimal, otherwise ethylene oxide may persist and will inhibit streptococcal growth. In this laboratory we have always sterilized milk and other media in Erlenmeyer flasks containing no more than 2/5 of their capacity of fluid to be sterilized. If the flask is filled more than this, the alteration in ratio of exposed surface to fluid volume causes a delay in the escape of ethylene oxide, and residual ethylene oxide may persist past the 24 hour period of sterilization.

The susceptibility of the various streptococcal strains to lactenin is a graded character. Some strains die out in its presence; the early growth of others is retarded, but full growth is ultimately achieved; and still others appear to be completely unaffected by it, growing as well, from the beginning, in fresh as in boiled milk. When the effect of lactenin on growth is observed after 24 hours, particularly in the method employing milk-agar plates, the intermediate degrees of susceptibility are not seen and with few exceptions the streptococci fall into two classes: those which are fully inhibited by it and those which grow well in its presence. The first class is regarded as "sensitive" to lactenin and the second class as "resistant."

The distribution of lactenin-sensitive and lactenin-resistant strains among the several serological groups was not at random. All group A strains were sensitive to lactenin, regardless of serological type. Most group B, C, D, and

E strains were resistant. Strains of groups F, G, H, K, and L fell into both classes. Our early hope that we could tell whether an unknown streptococcus belonged to group A or did not belong to group A by testing it for sensitivity to lactenin was not borne out by more extensive experience. Although the testing of 453 strains of hemolytic streptococci in the field gave an error in this correlation of only slightly more than 1 per cent, there are some practical difficulties in the performance of the test which would tend to make the error much higher than that under some circumstances. One difficulty arises from failure to distinguish between beta and alpha streptococci. If a green or alpha prime streptococcus is mistakenly considered to be beta hemolytic on primary isolation, which can occasionally happen, and is tested by the lactenin sensitivity method, it can easily be misidentified as a group A streptococcus, since many of the green streptococci are inhibited by lactenin. Such an error would be avoided if serological identification were used. Another difficulty arises from failure to use pure culture in testing. The inoculum used in streaking the plates usually results in a solid wedge of growth on the milk and blood agar plates. It is possible for a few contaminating colonies to be concealed in this massive growth of group A streptococci and to grow unrecognized on the blood agar plate, although good growth of the contaminant may occur on fresh milk agar plates and be mistakenly considered streptococcal growth.

The desire to differentiate organisms of group A from the other groups by technics simpler than the standard serological methods has led to several proposed cultural procedures. One of these (11) depended on the appearance of streptococci streaked on maltose–horse blood agar. Another (12) depended on the appearance of colonies in semi-solid serum agar and on the presence or absence of opalescence when the organisms were grown in 10 per cent serum broth. Another (13) attempted to differentiate streptococci of the various groups on the basis of characteristic patterns of hemolysis in blood agar. We have tried all these methods, and have found them to be inadequate for the necessary differentiation among the groups. The lactenin-inhibition test is, perhaps, superior to any of the others, but nevertheless it is subject to distinct error and we hesitate to recommend it for the purpose. The situation at present remains that there is no simple cultural method which can be substituted for the precipitin reaction in determining streptococcal groups.

If the lactenin of human milk inhibited group A streptococci and the lactenin of cow milk inhibited group B streptococci it could be reasoned that the formation of lactenin had arisen, from an evolutionary point of view, as a result of contact between the host and the commonest infecting streptococcus of the host. We have shown, however, that the lactenins of the 2 species and of goats, too, attack the same streptococcal strains. This suggests that the formation of lactenin is a general function of mammary tissue unrelated to infectious occurrences.

SUMMARY

The measurement *in vitro* of lactenin, the antistreptococcal substance of milk, is affected by the size of the inoculum, the temperature of incubation, and the type of medium employed.

Hemolytic streptococci belonging to the several serological groups vary in susceptibility to lactenin. All group A streptococci, regardless of type, are highly sensitive to it, and milk receiving a small inoculum sterilizes itself within 48 hours or less. By contrast, most strains of groups B, C, D, and E, although they may temporarily be inhibited, ultimately achieve full growth. Strains belonging to groups F, G, H, K, and L vary in sensitivity, some being fully inhibited and others achieving full growth. When streaked on the surface of milk-agar plates and examined at the end of 24 hours the streptococci fall into two classes: sensitive strains which do not produce visible colonies on the plate, and resistant strains which grow excellently. Very few strains show an intermediate degree of sensitivity.

Human and goat milk contain an antistreptococcal principle which appears to be the same as the lactenin of cow milk, since streptococci which are inhibited by milk from one species are inhibited by milk from the others, and *vice versa*.

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