THE SOURCE OF AGGLUTININS IN THE MILK OF COWS.

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For several years the milk of cows has been under investigation in this Department with special relation to the udder as a locus of multiplication of *Bacillus abortus* and the bearing of this condition on infection of the placenta during gestation. The presence of this organism in the udder has been detected by inoculating guinea pigs with the concentrated sediment of milk and by plating portions of the same. At the same time the samples of milk have been subjected to the agglutination test. In the majority of cases the agglutinins in the milk presented that fraction of the blood agglutinins which has been usually ascribed to a filtration from the blood. Certain cases, however, could not be ranged in this group. The ratio of agglutinins in the milk to that of the blood serum was far higher than that due to such assumed filtration. Thus in the first class of cases this ratio fluctuated between 1/16 and 1/64. In the second it ranged from 1/4 to 1/1. In samples of colostrum taken soon after parturition it ranged from 1/1 to 2/1. Here the high concentration is in part due to a slow accumulation in the inactive udder for within a few days after the active secretion of milk has begun the agglutinins may fall to the 1/16 or 1/32 ratio. In a few cases they have remained high. Two instances taken from experiments made for another purpose illustrate the differences which may be encountered (Table I). Both cows were vaccinated by a subcutaneous injection of living bacilli. Although both reacted to the inoculation with an accumulation of agglutinins up to the same high level in the blood serum, the milk showed in one case a low output, in the other a high output of

TABLE I.

Cows A843 and 585. Vaccinated with Living Bacilli May 7, 1921.

Date of blood sample.	Blood serum titer.	Date of milk sample.	Milk titer.
Cow A843.			
1921		*1921*	
		Apr. 21	1:20
May 1	1:40		
" 10	1:40	May 10	1:20−
" 17	1:1,280+	" 17	1:20
		" 24	1:20
" 31	1:2,560	" 31	1:20
		June 7	1:20
June 22	1:1,280+	" 21	1:20
		July 11	1:20−
		Sept. 26	1:10
Nov. 8	1:1,280	Nov. 16	1:20−
1922		*1922*	
Jan. 25	1:320+	Jan. 25	1:40−
Cow 585.			
1921		*1921*	
		Apr. 21	1:20
May 1	1:40		
" 10	1:80−	May 10	1:20−
" 17	1:1,280	" 17	1:40+
		" 27	1:40
" 31	1:2,560	" 31	1:40
		June 7	1:20
June 22	1:1,280		
		July 11	1:40
		Sept. 26	1:320+
Nov. 8	1:1,280+	Nov. 18	1:640
		Dec. 12	1:640
1922		*1922*	
		Jan. 6	1:1,280
Jan. 25	1:1,280	" 25	1:640

agglutinins. The stage of lactation was about the same in both animals.

The second animal (No. 585), a cow weighing about 1,000 lbs. and secreting about 28 lbs. of milk on January 6, was discharging daily

through the udder fully one-third of the agglutinin content of the blood. The only demonstrated difference between the two cases was the presence of living *Bacillus abortus* in the milk of No. 585. A large series of cases tested on guinea pigs has shown that a low agglutinin titer of the milk is not associated with *Bacillus abortus* in the udder, whereas from many animals with a high agglutinin content, *Bacillus abortus* was isolated from the milk through guinea pigs. In the few negative cases probably repeated tests would have revealed the bacilli.

Two alternatives presented themselves to be examined experimentally. Either there is a local production of agglutinins in the udder tissue or else the udder tissue is made completely permeable to the blood agglutinins in the presence of *Bacillus abortus*.

EXPERIMENTAL DATA.

To interpret the effect of injecting bacteria, dead or living, into the udder ducts the relation existing among the different parts of the udder through the ducts should be kept in mind.

The udder of the cow is divided into two anatomically independent lateral halves, bound closely together by connective tissue. They arise from two separate anlages in the embryo. Each half is divided into two quarters and each quarter has its group of ducts and its own teat canal. The parenchyma of each pair consisting of fore and hind quarter shows no dividing line but appears to form a single secreting mass. The quarters are, however, relatively independent, for infectious processes are, as a rule, restricted to one quarter at the start. In what follows concerning the local production of agglutinins it is of importance to know whether fore and hind quarters are in communication, even though restricted, with one another through their ducts. To throw light on this a pathological reaction was brought into play. When suspensions of *Bacillus abortus* are injected into a quarter there is a prompt reaction in that quarter, manifested locally by swelling of the entire quarter and a copious emigration of polynuclear leucocytes into the milk. This is at its maximum within 24 hours.

A cow about to be slaughtered, No. 601 of the experiments to be detailed, received a suspension of *Bacillus abortus* into the left fore quarter at 4 p.m. The cow was killed next day at 3 p.m. and the udder removed and portions of the four quarters fixed in Zenker fluid.

The microscopic examination of the injected quarter showed a very extensive filling up of the secreting alveoli with polynuclear leucocytes. The same kind of cells permeated the epithelium and the interstitial tissue where this was appreciable in extent. The other quarters, including the left hind quarter, showed some slight focal infiltration which is probably to be accounted for by the presence of staphylococci and streptococci found in earlier studies of this case. It is highly probable, therefore, that if any communication exists between the ducts of the fore and hind quarters it is very restricted.

The suspension was injected into an udder duct as follows: A milking tube was chosen long enough to pass through the teat canal into the cistern. To this a glass syringe holding 25 cc. was attached by means of a rubber tube. This outfit was sterilized. The udder was washed and left partly moist. After the introduction of the milking tube, some milk was allowed to drain out and then the culture injected. The teat canal was compressed for 5 minutes to prevent escape of the fluid.

Because of the character of the milk it cannot be used in its native condition for agglutination tests since it is impossible to distinguish in tubes in the usual way the clumping in low dilutions. To clarify the milk rennet has been found satisfactory. Unless the cream is taken off first, the whey which separates out after clotting with rennet is clouded. Therefore, the milk is centrifuged and the cream removed. The tube of milk is then put into a water bath at about 35°C. A rennet tablet is dissolved in about 10 cc. of sterile distilled water and 8 to 10 drops added to the slightly warmed milk, the whole shaken and left standing for 10 or 15 minutes to coagulate. It is then put in a water bath at about 45-50°C., which causes the coagulum to shrink and a water-clear whey to separate out. This whey is used for the agglutination test as is blood serum and the dilutions made with normal salt solution.

I. The Injection of Living Bacilli into the Udder.

The first experiment attempted was to inject a suspension of living bacilli directly into the ducts of one quarter of the udder to verify inferences based on spontaneous cases.

Cow 523.—Calved July 9, 1919, and Sept. 2, 1920. In October she reacted to the tuberculin test.

Agglutinin tests on record before the experiment was started were: Oct. 22, for the blood somewhat above 1:40, for the milk on Oct. 22 and Nov. 8 about 1:10.

The flora of the udder was looked into before inoculation. On a blood agar plate colonies of hemolytic streptococci appeared which, treated with an immune serum towards bovine hemolytic streptococci, were agglutinated completely at 1:1,280. There were about 17,600 in a cubic centimeter of milk. Only a small per cent of colonies of other bacteria were on the plate.

On Nov. 8, 1920, after having been milked out the right fore quarter was inoculated by an injection into the milk duct of 18 cc. of a suspension prepared as follows: *B. abortus* isolated Oct. 28 from the meconium of a fetus (No. 460) was cultivated on a layer of agar in a Blake bottle sealed with sealing wax for 3 days at 37°C. The growth was washed off, suspended in normal saline, and diluted to a density of 2.4 as measured by the Gates instrument. This dilution corresponded to approximately 3,300 million bacteria per cc.

Considerable attention was given both during milking and in the intervals to prevent contact infection of the other quarters. After several weeks this care was discontinued because of the greater opportunities for infection by way of the blood.

Next day, the cow was uneasy and refused her food. The temperature at 7 a.m. was 41°C., at 1 p.m. 41.6°C. The *R. F.*[1] was swollen to twice its normal size. It was warm and sensitive to manipulation. The fore milk was thick and flaky. The total secretion of milk fell from 9 to 3 quarts. The agglutinin titer of the milk had not risen.

Plate cultures of the milk showed a decided diminution of streptococci in the injected quarter. Compared with the colonies obtained before inoculation they had been reduced 400 times. It was at first supposed that the injection of *B. abortus* had cleared the way for a streptococcus mastitis, but the platings showed, on the contrary, that the mastitis was due to *B. abortus*.

On Nov. 10, the cow was not eating the normal amount of feed. The temperature at 7 a.m. was 40°C., and at 1 p.m. 40.6°C. The injected quarter was still swollen and firmer than yesterday. The swelling was extending forward. The fore milk was thick and yellow. The total yield was 4 quarts.

On Nov. 11, the general condition of the cow and udder was the same but the temperature was lower, 39.2°C. at 7 a.m. and 38.6°C. at 5 p.m. Samples of fore, middle, and last milk from the four quarters were tested and since the difference in agglutinins was inappreciable only the middle milk was tested. The *R. F.* was now 1:80, the L. F. 1:20, the others below 1:20. The blood titer was between 1:40 and 1:80.

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[1] R. F., R. H., L. F., and L. H. stand for right fore and right hind quarter and left fore and left hind quarter respectively. The italics indicate the injected quarter.

On this day a distinct increase in the number of hemolytic streptococci in the injected quarter was evident. There were, per cc., 1,105, 920, and 250 colonies in the fore, middle, and last milk respectively.

On Nov. 12, the cow was not eating normally. The udder was swollen, the milk not so thick, and the total more abundant.

On Nov. 14, there was slight improvement in all directions. The titer of the milk was as follows: *R. F.* 1:80, the rest 1:20. Colonies of hemolytic streptococci per cc. were distributed as follows: *R. F.* 350, L. F. 230, R. H. 1,730, L. H. 4,020.

The milk titer on the 10th and the 11th day showed an increase in agglutinins towards *B. abortus*:

	Nov. 18	Nov. 19
R. F.	1:320	1:640
R. H.	1:80	1:320
L. F.	1:160	1:320
L. H.	1:80	1:320

Guinea pigs inoculated with last milk of *R. F.* Nov. 19 subsequently proved negative to *B. abortus*.

On Nov. 21, the only abnormalities observed were the still swollen quarter and its reduced secretory activity. The agglutinin titer was as on the 19th. Unfortunately the agglutination test of the blood had not been taken since Nov. 11 when it was not quite 1:80. On Nov. 21 it was 1:1,280, having risen to this high level in the meantime.

On Nov. 29, the blood titer had risen but that of the milk had declined. The blood titer was 1:2,560, *R. F.* 1:160, the rest 1:80. The hemolytic streptococci were gradually gaining ground: *R. F.* 6,550, R. H. 12,150, L. F. 6,520, and L. H. 7,620 colonies per cc.

On Dec. 9, 31 days after the inoculation, the milk titers had again risen slightly: *R. F.* 1:320, the rest 1:160. On Dec. 16, the milk titers were as on Dec. 9. On Dec. 27, 49 days after inoculation, *R. F.* was still indurated and not secreting as much as before the inoculation. The milk titers shifted somewhat in that the injected quarter rose and the others fell: *R. F.* 1:640, the rest 1:80. The blood titer was high, 1:2,560.

On Jan. 14, 1921, the 67th day of the experiment, the udder conditions were as before. The milk titers shifted slightly: *R. F.* 1:640, R. H. 1:80, the others 1:40 or less. The blood titer was 1:2,560. The colonies of hemolytic streptococci were still increasing in numbers: *R. F.* 7,800, R. H. 9,600, L. F. 9,000, and L. H. 14,200 per cc.

On Jan. 26, the 79th day of the experiment, the milk titers were: *R. F.* 1:320, the rest 1:40. The colonies of hemolytic streptococci per cc. were as follows: *R. F.* 8,900, R. H. 4,600, L. F. 900, and L. H. 10,400. Two guinea pigs inoculated with milk from the injected quarter were found free from *B. abortus* later on.

Second Injection.—On Jan. 27, a similar injection of a living *B. abortus* sus-

pension was made into the left fore quarter of the udder. The cow reacted during the following 24 hours much as before both as regards the general condition and the udder itself.

On Jan. 28, the agglutinins in the injected quarter had risen promptly: *L. F.* 1:160, *R. F.* 1:640, R. H. 1:40, L. H. 1:40. At the same time the agglutinins in the quarter injected first rose again and maintained a high level throughout.

The hemolytic streptococci had shifted as a result of the injection.

	Before injection.	After injection.
L. F.	900	0
R. F.	8,900	11,800
R. H.	4,600	1,700
L. H.	10,400	15,700

They had practically disappeared from the injected quarter in which an acute mastitis developed.

On Jan. 29, 2 days after the injection, the cow still refused food and the injected quarter was twice its normal size, firm, painful, and warm to the touch. The fore milk was thick and yellow.

The agglutinin titer was higher: *L. F.* 1:320, *R. F.* 1:640, the others 1:160. The hemolytic streptococci were reappearing in the injected quarter and were present in the right fore quarter injected first in larger numbers than ever: *L.* F. 300, *R. F.* 30,100, R. H. 4,500, L. H. 20,100 colonies per cc.

On Jan. 31, 4 days after the injection, the titers were as follows: *L. F.* 1:640, *R. F.* 1:640, R. H. and L. H. a trifle below 1:80; the blood titer was 1:2,560.

On Feb. 6, the cow's appetite was improving. The injected left fore quarter was still large and indurated and the fore milk thick and yellow. The titers were slightly lower: *R. F.* and *L. F.* 1:320, R. H. and L. H. 1:80.

Four later determinations with middle milk were made 11, 14, 20, and 25 days after the second injection. In the injected quarters the titers fluctuated between 1:640 and 1:320. In the other quarters the titer gradually rose to 1:320 on the 14th day in R. H., which then fluctuated between 1:160 and 1:320. L. H. reached a titer of 1:320 on the 20th day and remained there at the next test. The titer of the blood was 1:5,120 on the 14th day and continued there during the remaining tests.

The hemolytic streptococci again disappeared from the plates from the injected left fore quarter on the 4th day and did not return during the remaining tests although they were present in abundance in the other quarters.

On Jan. 26, two guinea pigs received into the peritoneal cavity each 5 cc. of sedimented and centrifuged milk from *R. F.* Neither guinea pig showed any lesions after 3 weeks and cultures from the spleen were negative.

The cow was killed Feb. 23, and the udder reserved for gross and microscopic examination. The only tuberculosis lesion found in this positively reacting cow was in the left bronchial lymph node. The focus was the size of a walnut and partly calcified. The udder presented no lesions recognizable with the

unaided eye and palpation after the organ had been cut into thin slabs. Sections of fixed and hardened tissue from both inoculated fore quarters and the right hind quarter as a control were examined. In all certain lesions were present. Acini, singly and in smaller and larger groups, were filled loosely with polymorphonuclear cells and some acidophils. Each cell of the secreting epithelium in these foci has one large vacuole (fat). The interstitial tissue shows slight infiltrations with lymphoid and plasma cells.

In one section of the left fore quarter there is a focus, occupying two-thirds of a lobule, in which the acini are compressed by endothelial cell accumulations resembling lesions in the guinea pig due to *B. abortus*. The surrounding acini show a modified vacuolated epithelium. The presence of hemolytic streptococci in this udder renders it impossible to ascribe definitely the diffuse lesions to either *B. abortus* or streptococci.

The details of this experiment may be briefly summarized. The injection of living bacilli into the right fore quarter produced a very prompt reaction characterized by febrile disturbances lasting about 2 days and a mastitis shown by marked swelling, heat, and tenderness of the injected quarter, and changes in the physical appearance of the secretion. The swelling lasted a number of weeks.

The agglutinins rose in the injected quarter slightly after 3 days and markedly after 10 days. They rose slightly in all the other quarters after 10 days. The blood was high on the 13th day (not examined earlier).

A second injection into the left fore quarter produced in an accelerated manner all the above phenomena. The agglutinins rose not only in the injected quarter but equally in the right fore, previously injected, and somewhat in the two remaining quarters within 2 days. The blood titer continued to rise and remained high throughout. Injection of milk into guinea pigs failed to demonstrate living bacilli in the inoculated quarters.

Incidentally, the hemolytic streptococci parasitic in the udder disappeared for a time after each injection from the inoculated quarter.

The tentative inference to be drawn from this experiment was a local production of agglutinins in the inoculated quarters. The appearance in the other quarters and in the blood may be ascribed to the dissemination and multiplication of the bacilli both locally in the udder and generally in the system.

II. The Effect of Cultures of Bacillus abortus Killed by Heat after Injection First under the Skin and into a Vein and Then into the Udder.

The first experiment did not prove clear-cut owing to the opportunity for the dissemination of the living bacilli. An experiment with killed cultures had been going on at the same time. It was first attempted to define the relation of the milk to the blood agglutinins by treating a cow with subcutaneous injections to note the effect on the udder agglutinins and then inject heated bacilli into the udder directly.

Cow 487.—Gave birth to a full-term, female calf, slightly under weight, on Mar. 30, 1920. The placenta was discharged *in toto*. There were no indications of disease except for the presence of scattering villi in the cotyledons which were tipped with whitish calcareous incrustations. The cow had reacted to tuberculin.

The blood agglutinins had been tested before the animal was used for the experiment on the following dates and with agglutinin limits as indicated: Nov. 25, 1919, 1:10+, Jan. 6, 1920, 1:20, Jan. 30 and Feb. 25, 1:80. On the day of calving (Mar. 30) the serum titer was 1:160, the colostrum titer 1:320. The serum titer was found to be 1:40 on Oct. 13 and 22. The titer of the mixed milk on the day following parturition was only 1:20. The same titer was found July 13 and Oct. 22.

On Nov. 4, 1920, the cow received subcutaneously 10 cc. of a suspension of *B. abortus* killed by heat, equivalent to 30 cc. of standard turbidity. The strain of *B. abortus* was the same as that used in Experiment I.

The vaccine was prepared by growing the culture on standard agar in a Blake bottle, sealed with sealing wax and incubated at 37°C. for about 72 hours. The growth was washed off in 0.85 per cent NaCl solution and the density reduced by means of the Gates instrument to 2.4. The bacilli were killed in a sealed tube immersed in a water bath at 62°C. for 25 minutes. The heated suspension was incubated over night to allow for any multiplication of surviving bacilli, then two agar slants were inoculated with 2 drops of the suspension, sealed, and incubated to test the sterility of the vaccine.

In the course of the following 6 days, a slight swelling appeared locally and the temperature rose to 39.1°C. and fell again. On Nov. 10, the blood titer had risen to 1:320. The titer of the milk of the four quarters was 1:10. On Nov. 14, the titer of milk from the right quarters was 1:10, from the left 1:20. On Nov. 17, 12 days after the injection, the blood titer was up to 1:640, the milk titer back to 1:10. On Nov. 29, the blood was somewhat below 1:640, the milk titer remained the same.

A second subcutaneous injection of heated bacilli was given Dec. 4, on the opposite (right) side of the neck. The dose was the same as the first.

During the next 5 days, the local swelling appeared a trifle larger and more sensitive than that following the first dose. On Dec. 9, the blood titer was 1:320, the milk titer also a trifle lower than heretofore. Possibly the cow was passing through a slight "negative" phase. On Dec. 16, the 12th day after the second injection, the blood titer had risen to nearly 1:1,280, the milk titer was again 1:20.

On Dec. 20, both titers were as on Dec. 16. On this day a third injection of heated bacilli was given on the right side of the neck, anterior to the site of the first injection. The dose was 8 cc. of a six times normal density. The local swelling this time reached the size of a large hen's egg and remained so for several weeks. 3 days after the injection a conjunctivitis of the right eye appeared with clouding of cornea which cleared up after several weeks.

On Dec. 27, the blood titer was 1:1,280, that of the milk was a trifle above 1:20. On Dec. 31, the milk titer was 1:40. On Jan. 6, the milk titer was 1:40 and the blood titer 1:1,280. On this day, 17 days after the last injection, a dose of 5 cc. of a triple standard suspension of heated bacilli was injected into a jugular vein. Within 2 hours the respirations had increased in number. Within 4 hours the animal had a severe chill. The temperature rose to 41.2°C. On the following day the cow refused part of her food and the milk secretion was reduced. Next day conditions had returned to normal. On Jan. 14, 8 days after the intravenous dose, the blood titer had risen to 1:2,560. The fore milk was 1:20, the middle and last milk 1:40.

The various injections made thus far had produced a relatively high agglutinin titer of the blood serum, even higher than is usually found in infected cows. However, the titer of the milk had risen only very slightly.

On Jan. 21, 18 cc. of a standard suspension of *B. abortus*, killed by heat, were injected directly into the duct of the right fore quarter of the udder. Within 12 hours the cow became quite sick in appearance. The respirations were rapid and the milk secretion reduced one-fourth. The injected quarter was swollen and warm, the secretion thicker than normal and diminished in quantity. On Jan. 23, the injected quarter was still swollen. The agglutinins in the middle milk from two quarters were increased: *R. F.* 1:160, R. H. 1:80, L. F. and L. H. 1:40. On Jan. 24, the injected quarter was still swollen and warm, the fore milk thick and yellowish. Milk titers the same as yesterday. On Jan. 26, the fore milk of the injected quarter had cleared up but the quarter was still a little swollen. The milk titers had dropped slightly.

On Jan. 28, the titers were as follows: *R. F.* 1:160, the rest 1:40. On Jan. 31, 10 days after the injection into the udder, the milk titer of the injected quarter was still 1:160, that of the three other quarters had risen to 1:80. The blood titer was 1:2,560. On Feb. 2, the titer of *R. F.* milk was up to 1:320, that of the rest about 1:80. On Feb. 7, the titer of the *R. F.* milk was still higher, 1:640, and the others had risen to 1:320.

On Feb. 10, the milk titer was unchanged. The blood titer was not 1:5,120. On Feb. 11, the titer of *R. F.* was still 1:640. On this day five guinea pigs received into the peritoneal cavity each about 5 cc. of milk sedimented and centrifuged

from 1 liter taken from the right fore quarter. None showed any enlarged spleen after 6 weeks, nor were cultures positive for *B. abortus*. The test was to determine whether any living bacilli had invaded the udder. On Feb. 16, there was a slight decline in the milk titers. The *R. F.* titer was 1:320, the rest 1:160.

As late as Feb. 21, 31 days after the injection into the right fore quarter, this part seemed a little large and the amount of milk secreted below that of the pre-inoculation period. On this day the milk titers were as before.

The cow was killed Feb. 24. The autopsy showed five nodes in the dorsal mediastinal chain containing caseous and calcareous foci. The lymph nodes were up to a walnut in size. The udder was obtained and thoroughly dissected. Macroscopic lesions were not detected. In two of four sections of *R. F.* there were several areas in which five to six contiguous acini were filled with polynuclear cells. In the interstitial tissue occasional foci of lymphocytes.

The experiment may be summarized as follows: A cow received three subcutaneous and one intravenous injection of a heated suspension of *Bacillus abortus*. As a result the agglutinins of the blood rose from 1:40 to 1:2,560 in 71 days, the titer of the milk from 1:10 to a feeble reaction at 1:40.

On the 78th day, a heated suspension of *Bacillus abortus* was injected directly into the cistern of the right fore quarter of the udder. The agglutinins rose within 2 days in the injected quarter and after 10 days in the others. The titer slowly rose in the injected quarter to 1:640 where it remained until the 20th day when it fell slightly. A similar but less pronounced curve was followed in the other quarters, the maximum being 1:320.

The repeated injection of killed bacilli thus led to a concentration in the blood serum of agglutinins as high as or even higher than that which appears in the spontaneous disease.

The udder agglutinins rose but very little as a result of this preliminary treatment. On the other hand, the direct injection of killed bacilli into the ducts of one quarter of the udder led to a considerable rise in the agglutinins of the milk of that quarter and to a moderate rise in the other quarters.

III. The Direct Exclusive Injection of Dead Bacilli into the Udder Ducts.

In the four following cases the heated bacilli were injected into the udder ducts without any preliminary injection into the subcutis or veins.

No. 584.—Eastern cow, purchased Mar. 22, 1921. Second or third calf born Jan. 5, 1921. Three guinea pigs which received sedimented and centrifuged milk from the udder just before the first injection were negative as to *B. abortus*.

The experiment comprised two injections of 16 cc. each of a standard suspension of heated bacilli (3,300 million per cc.), the first into the right fore quarter, the second into the right hind quarter, 31 days after the first.

There was the usual effect on the udder and its secretion. The injected quarter became swollen to nearly twice its normal size within 18 hours and slowly shrank but it did not regain its original volume in 4 months. After the second injection, the right hind quarter became enlarged in the same way. The fore milk after the first injection was thick and viscid for 5 days. The total milk secretion fell from about 18 to 14 lbs. in 3 days. On the 10th day it was again 18 lbs. The secretion after the injection of the right hind quarter fell from $18\frac{1}{2}$ to $13\frac{3}{4}$ lbs. in 3 days. On the 6th day it was $16\frac{3}{4}$ lbs. A slight febrile reaction accompanied each injection and lasted about 24 hours.

The changed physical condition of the milk following the injection was associated with a marked cellular emigration as was the case in the preceding experiments. Following the second injection a count of the leucocytes was carried out. In Table II the number of cells per cubic centimeter and the corresponding limit of the agglutinin titer are given. It will be noted that the maximum cell output occurred within 24 hours after the injection, whereas the agglutinin titer did not rise until the 10th day when the cell content had fallen to the pre-inoculation level. The cell content of *R. F.*, L. F., and L. H. before the second injection was 2 million, 20 million, and 12 million per cc. respectively.

The agglutinin content of the milk and blood serum during the period of observation is best shown in Table III.

It will be noted that, following the first injection, the agglutinins of the affected (*R.F.*) quarter rose slightly on the 2nd and again on the 8th day and on the 29th day to a maximum of 1:320. The L. F. and the L. H. also showed increases slightly above what should be expected as coming from the blood. The R. H. remained unaffected. The blood in the meantime rose to a slightly higher level (1:640).

After the second injection the affected (*R. H.*) quarter developed a relatively high agglutinin content with a maximum of 1:640 on the 19th and 21st days. At the same time, the quarter injected first (*R. F.*) not only continued its high content of 1:320 but rose to 1:640 on several occasions. A relatively high agglutinin output (1:320) was found as late as September 15, nearly $4\frac{1}{2}$ months after the second injection. On the other hand, the two "control" quarters remained fairly low (1:40) throughout. The blood titer at this date had dropped to 1:160.

The bacterial flora was examined before the first injection of the udder. A hemolytic staphylococcus was the chief species. At times other types were present. The marked difference in numbers of colonies on blood agar plates in fore, middle, and last milk makes the meager examinations yield inconclusive results and the figures are therefore omitted.

TABLE II.

Cow 584. Cellular Reaction Following Second Inoculation into the Udder.

Date.	R. H.	
	Agglutination titer.	Cell count.
1921		
May 2	+1:10	637,645
May 6	15 cc. of dead bacilli injected.	
May 7	±1:40	61,543,361
" 8	±1:40	47,193,796
" 9	±1:40	15,306,096
" 10	+1:20	3,826,524
" 12	+1:40	1,913,262
" 16	±1:320	656,631
" 20	±1:320	
" 25	+1:640	1,272,508
Sept. 8	+1:320	3,507,647

Cow 600.—Brought from the Middle West Nov. 20, 1920. Reacted to tuberculin. Received at the Department May 2, 1921. Milk tested on two guinea pigs for *B. abortus* with negative outcome.

On June 7, a standard suspension of a culture of *B. abortus*, killed by heat, was prepared and 15 cc. injected into the ducts of *R. F.* The effect on the udder was as described in the preceding cases. The fore milk was thick and viscid or "stringy." The total amount fell from 25¾ to 22½ lbs. on the 3rd day. On the 23rd day the total yield had risen to 25½ lbs.

On June 30, a second injection of the same dose into *L. F.* was followed by a much greater swelling of the treated quarter. The fore milk was very thick, tending to obstruct the teat canal. The total yield fell again from 25½ to 19 lbs. on the 2nd day. It then began to rise until on the 7th day it was 24⅜ lbs. Each injection was followed by a rise in body temperature to between 40° and 41°C. which lasted about 24 hours.

The changes in the agglutinins of the milk and blood are given in Table IV. Only middle milk was tested.

The reaction to the first injection was less pronounced than in former cases. The agglutinins in the treated quarter rose from less than 1:10 to 1:20 in 8 days and to 1:40 in 20 days. The other three quarters responded by a final rise from less than 1:10 to 1:20 in 20 days. The blood rose from 1:80 to 1:640 in the same interval.

After the second injection into the *L. F.*, the agglutinins in this quarter rose from 1:20 to 1:320 in 12 days, then fell slowly. $2\frac{1}{2}$ months after this injection the milk titer was still 1:80. At the same time the agglutinins in the quarter first injected (*R. F.*) began to rise from 1:40 and reached a maximum of 1:160 in 6 days. Here they remained for 20 days. $2\frac{1}{2}$ months after the second injection the milk from this quarter was still 1:40. One of the two untreated quarters reacted slightly to the second injection by a rise in the agglutinin titer from 1:20 to 1:40. The titer of the other quarter remained as it was after the first injection.

After the second injection the blood titer rose again from 1:320 to a peak of 1:1,280 in 19 days. It remained at or above 1:640 from the 9th to the 27th day after the injection when the tests were discontinued. After a period of $2\frac{1}{2}$ months from the second injection the blood titer stood at 1:160.

The cellular reaction following the injections which partly accounts for the thick, stringy fore milk closely resembled that observed in No. 584 and is shown in Table V. On the day following the first injection the fore milk contained a heavy deposit after sedimentation which consisted of cells, chiefly polynuclear. Only a few mononuclears were present, together with a blue-staining amorphous substance holding cells together. On the 2nd day a sample of middle milk had a heavy floating layer, made up chiefly of cellular elements. On the 3rd day a definite cell sediment or floating layer had disappeared.

Cow 601.—Introduced from the Middle West Nov., 1920. Giving milk at this time. Reacted positively to tuberculin and obtained for the experiment May 2, 1921. Two guinea pigs receiving 5 cc. of sedimented and centrifuged milk the day before the first injection were negative as to *B. abortus*.

Two injections of 15 cc. of a standard suspension of *B. abortus* killed by heat were made, one on June 29 into *R. F.*, and one on July 18 into *R. H.* There

TABLE III.

Cow 584. Injection of Dead Bacilli into the Udder Ducts.

Agglutination Tests.

Date.	Milk.																								Blood.							
	R. F.							R. H.							L. F.						L. H.												
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
1921 Apr. 5	16 cc. of dead *B. abortus* culture injected into right fore quarter. (Mixed milk 1:10.)																																	
Apr. 6	+	+	±	−	−	−		±	−	−	−	−			+	−	−	−	−		+	−	−	−	−								
" 7	++	+	+	±	−			+	+	−	−	−									++	+	−	−	−								
" 8	++	+	±	−	−			+	+	−	−	−			+	±	−	−	−		+	+	−	−	−		++++	++	++	+	+	±	−	
" 9	+	+	±	−	−			+	±	−	−	−			+	+	−	−	−		+	+	±	−	−								
" 11	+++	++	+	+	−	−		++	+	±	−	−			++	+	+	±			++	+	+	±	−		C.	C.	C.	++++	++	+	+	−
" 13	++++	++	+	+	±	−	−	++	+	±	−	−			++++	+++	++	+	±	−	+++	+++	++	+	−	−								
" 15	+++	++	+	+	−	−	−	++	+	−	−	−	−		C.	++++	+++	++	+	−	++++	+++	++	+	+	−	C.	C.	C.	C.	C.	+++	++	−
" 18	++	+	+	−	−	−	−	+	±	−	−	−	−		++++	+++	++	+	−	−	+++	++	+	+	−	−	"	"	"	"	"	+++	+	−
" 19																																		
" 21	++	+	+	−	−	−	−	+	−	−	−	−	−		+++	++	+	−	−	−	++	+	±	−	−	−								
" 25	++	++	+	−	−	−	−	+	−	−	−	−	−		++	+	+	−	−	−	++	+	+	−	−	−								
May 2	++++	++++	+++	++	+			+	−	−	−	−	−		++++	+++	++	+	+	−	++++	+++	++	+	−	−	C.	C.	C.	C.	C.	+++	+	−
" 4	++++	++++	++++	+++	++	+	−	+	−	−	−	−	−		++++	+++	++	+	±	−	+++	++	++	+	±	−								
May 6	16 cc. of dead *B. abortus* culture injected into right hind quarter.																																	
May 7	C.	++++	++++	+++	++	+	±	++	+	±	−	−	−	−	+++	+++	++	+	−	−	++++	+++	++	+	±	−								
" 8	"	C.	++++	++++	+++	++	±	++	+	±	−	−	−	−	++++	+++	++	+	−	−	++++	+++	++	+	−	−								
" 9	"	++++	++++	++++	+++	++	+	++	+	±	−	−	−	−	+++	++	+	±	−	−	+++	++	+	±	−	−								
" 10	++++	++++	++++	++++	+++	++	+	++	+	−	−	−	−	−	++	+	±	−	−	−	++	++	+	±	−	−	C.	C.	C.	++++	++	−	−	
" 12	++++	++++	++++	++++	+++	+	−	++	++	+	−	−	−	−	+++	++	+	−	−	−	+++	++	+	−	−	−	C.	C.	C.	++++	+	−	−	
" 13																																		
" 16	C.	C.	+++	+++	+++	++	+	++++	++++	++	++	+	±	−	+++	++	+	−	−	−	+++	++	+	±	−	−	"	"	"	C.	+++	+	−	
" 20	"	++++	++++	+++	++	+	±	C.	++++	++++	++	+	±	−	+++	++	+	−	−	−	+++	++	+	−	−	−	"	"	"	"	++	+	−	
" 23	++++	++++	++++	+++	++	+	−	++++	++++	+++	+++	++	+	−	+++	++	+	±	−	−	+++	++	+	−	−	−								
" 25		C.	++++	+++	++	+	−		++++	++++	+++	++	++	+	+++	++	+	−	−	−	++++	++	+	−	−	−	C.	C.	C.	C.	++++	+	−	
June 1	C.	"	++++	+++	++	±	−	++++	++++	++++	+++	++	+	−	++	++	+	−	−	−	+++	++	+	−	−	−	"	"	"	++++	±	−	−	
" 13	"	"	++++	+++	++	++	±	C.	++++	+++	+++	++	++	±	++	+	±	−	−	−	++	+	±	−	−	−	"	"	"	++++	±	−	−	
" 27	"	"	++++	++++	++	++	±	++++	++++	++++	+++	+++	++	+	++	+	±	−	−	−	+++	++	+	±	−	−								
" 30																											C.	C.	C.	+++	−	−	−	
July 12	C.	C.	C.	++++	+++	++	+	C.	C.	C.	++++	+++	++	+	++	+	−	−	−	−	++	+	−	−	−	−	"	"	"	++++	−	−	−	
" 26	"	"	++++	++	+	−	−	"	"	"	++++	+++	+	−													"	"	++++	+	−	−	−	
Sept. 8											++++	++	+	−																				
" 15	C.	C.	++++	++	+	−	−	C.	"	++++	+++	+	±	−	++	−	−	−	−	−	+	−	−	−	−	−								
" 17																											C.	C.	C.	++	±	−	−	

In the tables C. indicates complete agglutination and clearing of the fluid.

TABLE IV.

Cow 600. Injection of Dead Bacilli into the Udder Ducts.

Agglutination Tests.

Date.	Milk.																			Blood.						
	R. F.					R. H.			L. F.							L. H.										
	1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
1921																									
May 25	−	−	−	−	−	−	−	−	−		−	−	−	−		−	−	−	++++	++	+	−	−	−	−
June 7	15 cc. of dead bacilli injected into right fore quarter.																								
June 8	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−							
" 9	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++	−	−	−	−	−
" 10	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C.	++	−	−	−	−	−
" 11	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	"	++	−	−	−	−	−
" 13	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	"	++++	+	−	−	−	−
" 15	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	"	C.	++++	+	−	−	−
" 17	++	+	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	"	"	+++	++	+	−	−
" 21	++	+	−	−	−	+	±	−	+	+	−	−	−	−	+	±	−	−	"	"	C.	+++	++	+	−
" 27	+++	++	+	−	−	++	+	−	++	±	−	−	−	−	++	+	±	−							
June 29	15 cc. of dead bacilli injected into left fore quarter.																		C.	C.	C.	++++	++	−	−
June 30	+++	+++	++	+	−	++	+	−	+++	+	−	−	−	−	++	++	+	−							
July 1	+++	++	+	±	−	++	+	−	+++	++	++	+	−	−	++	++	+	−							
" 2	+++	++	++	+	−	++	+	−	+++	+++	++	+	+	−	+++	++	+	±	C.	C.	C.	++++	++	−	−
" 6	++++	++++	+++	++	+	++	+	−	++++	++++	++++	++	+	−	+++	+++	++	+	"	"	"	+++	+	−	−
" 8	++++	+++	+++	++	+	++	+	−	++++	++++	++++	+++	++	−	+++	++	+	±	"	"	"	++++	++	+	−
" 11	++++	++++	+++	++	+	+++	++	+	C.	C.	C.	++++	++	+	+++	++	+	−	"	"	"	++++	+++	++	−
" 18	++++	+++	++	+	+	+++	++	±	"	"	"	+++	++	±	+++	+++	+	−	"	"	"	C.	C.	+++	+
" 26	++++	++++	+++	++	±	+++	+	−	++++	++++	+++	++	+	−	++++	++	+	−	"	"	"	"	++++	++	−
Sept. 15	++	++	+	−	−	−	−	−	+++	++	++	+	−	−	−	−	−	−							
" 17																			C.	C.	++++	±	−	−	−

TABLE V.

Cow 600. Cell Counts and Agglutinin Limits in the Milk.

Date.	R. F.		R. H.		L. F.		L. H.	
	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.
1921								
May 25	—	956,631	—	4,145,401	—	319,127	—	798,449
June 7	15 cc. of dead bacilli injected into right fore quarter.							
June 8	±1:10	182,077,767	—	5,102,032	—	318,877	—	6,377,540
" 9	+1:10	83,545,774	—		—		—	
" 10	+1:10	8,928,556	—		—		—	
" 11	±1:10	4,145,401	—		—	956,631	—	
" 13	±1:10	2,869,893	—		—		—	
" 15	+1:20	31,887,700	—		—		—	
" 17	+1:20	3,188,770	±1:10		—		—	
" 21	+1:20	5,739,789	±1:20		+1:20		±1:20	
June 29	15 cc. of dead bacilli injected into left fore quarter.							
June 30	+1:80	21,045,882	+1:20	8,709,679	+1:20	122,129,891	+1:40	6,377,540
July 1	±1:80		+1:20		+1:80	107,461,549	+1:40	
" 2	+1:80		+1:20		+1:160	90,240,191	±1:80	
" 6	+1:160		+1:20		+1:160	6,058,663	+1:80	
" 8	±1:320		+1:20		++1:160	5,739,786	±1:80	
" 11	±1:320		+1:40		+1:320	1,913,262	+1:40	
" 26	±1:160	22,959,144	+1:20		+1:160	637,754	+1:40	

was the same febrile reaction and prompt swelling of the injected quarters already described. The milk was thick both times, but cleared up on the 4th day. The drop in the total daily milk output occurred each time with nearly complete restitution within a week. The agglutinin titer of the milk from the four quarters changed but little following the first injection. That of *R. F.* rose from less than 1:10 to 1:20. The same took place in L. H. The other quarters rose to a bare 1:10. The blood titer, however, rose in 15 days from 1:10 to 1:320.

After the second injection a similar inertia in the local and general production of agglutinins was observed. *R. H.* rose on the 8th day to 1:20. *R. F.* rose one step to 1:40 after the 8th day. The other quarters remained unchanged. The blood titer also did not rise higher.

The cellular reaction following each injection was prompt and pronounced, as shown in Table VI. The limiting agglutinin titer is also given. Both agglutinin determination and cell count were made on the same sample of middle milk collected as nearly as ascertainable after the quarter had been half emptied.

At the time of the first injection the L. H. (untreated) was in a state of inflammation as shown by the very high content of cells. Traumatism due to a fall was suspected as the cause, but no definite proof of this is forthcoming.

A bacteriological examination of the fore milk before the udder injection on May 25 gave the following result.

R. F. 1,190 colonies per cc. of hemolytic streptococci.
R. H. 70 colonies per cc. of staphylococci.
L. F. 140 colonies per cc. of staphylococci.
L H. 670 colonies per cc. of staphylococci.

On June 30, the day following the injection of *R. F.*, the bacteria content of the middle milk was as follows:

R. F. 20 colonies per cc. of hemolytic streptococci.
R. H. 440 colonies per cc. of staphylococci.
L. F. 900 colonies per cc. of both species.
L. H. 300 colonies per cc. of both species.

On July 6, 7 days after the injection, the bacteria content of the middle milk was as follows:

R. F. 600 colonies per cc. of both species.
L. H. Countless colonies chiefly staphylococci.

No further examinations of the udder flora were made. 92 days after the second injection a spontaneous mastitis appeared in the *R. H.* with a high cell count, 38 million, and a low bacterial count of 10 colonies per cc.

Cow 897.—Grade Holstein. Calved shortly before being shipped from the Middle West. Received Dec. 14, 1921. Cow weighs 990 lbs. Reacted to the tuberculin test Feb., 1922.

TABLE VI.

Cow 601. Cell Counts and Agglutinin Limits in the Milk.

Date.	R. F.		R. H.		L. F.		L. H.	
	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.	Agglutination titer.	Cell count per cc.
1921								
May 25	—	7,105,294	—	637,754	—	478,315	—	857,112
June 29	15 cc. of dead bacilli injected into right fore quarter.							
June 30	—	132,652,832	—	637,754	—	2,232,129	—	119,259,998
July 1	±1:10	43,367,272	—		—		—	69,196,309
" 2	—	11,160,695	—		—		—	15,306,096
" 6	±1:20	35,714,224	—		—		±1:10	2,869,893
" 8	+1:20	10,204,064	—		+1:10		±1:20	16,262,727
" 11	+1:20	39,859,625	±1:10		+1:10		+1:20	17,857,112
" 18, a.m.	+1:10	12,436,263	—	956,631	—	956,631	+1:10	14,987,219
July 18, p.m.	15 cc. of dead bacilli injected into right hind quarter.							
July 19	+1:10	10,204,064	+1:10	73,660,557	—	1,275,508	+1:10	11,479,572
" 20	+1:10		+1:10	100,127,378	—	637,754	±1:10	
" 22	+1:20	35,395,347	+1:10	14,030,588	—	956,631	+1:10	47,831,550
" 26	+1:20	12,117,326	+1:20	3,188,770	+1:10	2,232,139	+1:20	12,117,326

In March a preliminary bacteriological study of the udder secretion was made. A variety of bacteria were found in the milk, among them streptococci, staphylococci, and diphtheroid forms. The limiting agglutinin titer of the milk toward these types varied from 1:10 to 1:160. It was assumed as heretofore that these forms would have no direct influence on the production of *B. abortus* agglutinin. This at the outset was less than 1:10. This experiment differed from the preceding in that repeated injections of dead bacilli were made into the same quarter. The suspension was of the density used heretofore and the amount introduced through a milking tube about 20 cc. each time. The agglutinin titers of milk and blood are given in Table VII.

It will be noticed that the agglutinin production may be considered medium when compared with Nos. 584 and 487. The response of the udder, slow and slight after the first injection, is accelerated and quantitatively increased after the second injection. After the third injection the titer of the milk is a trifle above that of the blood. The response of the other three quarters is slight and perhaps largely due to the rise in blood agglutinins.

The leucocytic reaction appeared as heretofore in the next morning's milk and reached a maximum then. Following the first injection the sedimented mass of cells in next morning's milk was equivalent to 8 per cent of the volume of milk of the injected quarter (887 cc.). On the following day the cell mass was very slight. Only a few flakes were detected on the 3rd day. The same reaction followed the second and the third injection. The cellular sediment disappeared after the 2nd day. The relatively high percentage of cellular sediment amounting to 8 per cent of the total milk secretion is in part due to the large amount of fat taken in by the leucocytes which may represent one-half the total volume of the leucocytic deposit.

After the third injection of heated bacilli into *R. F.* two injections of normal salt solution were made into L. F. 12 days apart. A slight increase in specific agglutinins was detected in the milk. The agglutinin content of *R. F.* maintained its original high titer as shown in Table VII. No appreciable cellular reaction followed the injection of salt solution.

TABLE VII.

Cow 897. Injection of Dead Bacilli into the Udder Ducts.

Agglutination Tests.

Date.	Milk.																		Blood.					
	R. F.						R. H.				L. F.				L. H.									
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:10	1:20	1:40	1:80	1:20	1:40	1:80	1:160	1:320	1:640
1922																								
Mar. 20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	+	±	−	−	−
Mar. 23	20 cc. of dead bacilli injected into right fore quarter.																							
Mar. 24	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
" 25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
" 27	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	±	−	−	−	−
" 29	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
" 31	+++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	++	+	−	−
Apr. 3	++++	++	+	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	C.	C.	C.	++++	++	±
" 7	++	+	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	"	"	++++	++	+	±
" 11	++	+	−	−	−	−	+	−	−	−	+	±	−	−	±	−	−	−						
Apr. 17	20 cc. of dead bacilli injected into right fore quarter.																							
Apr. 18	++	+	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−						
" 19	++	+	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−						
" 21	++++	++++	++++	++	+	−	+	−	−	−	+	−	−	−	+	−	−	−						
" 22																			C.	C.	++++	++	+	−
" 25	C.	C.	C.	++++	+++	+	++	+	+	±	++	+	+	±	++	+	+	±						
" 28	"	"	++++	++	+	−	++	+	−	−	++	+	−	−	++	+	±	−	C.	C.	C.	++++	++	−
May 1	"	"	++++	+	±	−	+	±	−	−	+	±	−	−	+	±	−	−						
" 4	"	"	+++	++	+	−	+	±	−	−	+	±	−	−	+	±	−	−						
" 9	"	++++	+++	++	+	−	+	−	−	−	+	±	−	−	+	−	−	−						
May 11	20 cc. of dead bacilli injected into right fore quarter.																		C.	C.	C.	+++	+	−
May 12	C.	C.	+++	+	−	−	+	−	−	−	+	±	−	−	+	−	−	−						
" 13	"	"	++++	++	+	−	+	±	−	−	+	±	−	−	+	−	−	−						
" 15		"	C.	+++	+	±	+	±	−	−	+	−	−	−	+	−	−	−	C.	C.	C.	+++	±	−
" 17	C.	"	"	++++	+++	+	+	±	−	−	+	±	−	−	−	−	−	−						
" 22	"	"	"	C.	+++	+	++	+	+	−	++	+	±	−	++	+	+	−	C.	C.	C.	++++	+	−
" 29	"	"	++++	++++	++	±	++	±	−	−	++	+	−	−	++	+	−	−						
May 31	20 cc. of normal salt solution injected into left fore quarter.																		C.	C.	C.	++++	++	−
June 2	C.	C.	++++	+++	++	−					++	+	−	−	+	±	−	−						
" 5	"	"	++++	+++	++	+	++	±	−	−	+++	+	±	−	+	±	−	−						
" 12	"	"	++++	++	+	±	+	±	−	−	+++	+	±	−	+	±	−	−						
	20 cc. of normal salt solution injected into left fore quarter.																							
June 13	C.	C.	++++	++	+	+	+	+	−	−	+++	+	+	−	+	+	−	−						
" 14	"	"	++++	+++	++	+	+	±	−	−	++	+	±	−	+	±	−	−						
" 16							+	−	−	−	+++	++	±	−	+	−	−	−						
" 23	C.	++++	+++	++	+	−	+	−	−	−	+++	++	+		++	+								
" 26	"	++++	+++	++	+	−	+	−	−	−	++	+			±									

DISCUSSION.

The relation between agglutinins in the blood serum and those in the milk of an actively secreting udder when suspensions of *Bacillus abortus* killed by heat are injected into the subcutis or the circulation directly is much the same as that observed in the spontaneous infection (Experiment II). The agglutinins in the milk occur rarely above 1:40 or below 1:10 and the ratio of content in milk to that in blood fluctuates between 1/32 and 1/64. When the udder is invaded and made the site of multiplication by *Bacillus abortus* then the agglutinins in the milk rise so as to shift the ratio to 1/4, 1/2, or even 1/1 (Experiment I).

So far as is known, the invasion of the udder by *Bacillus abortus* means its presence and multiplication in the milk in the acini and ducts and not in the connective tissue stroma. The condition of the udder tissues within 24 hours after the introduction of dead bacilli into the ducts and later, after subsidence of the acute reaction, indicate this. In other words, the residual milk in the acini and ducts rather than the udder tissue appears to be the seat of multiplication.

When bacilli killed by heat are injected into the udder ducts, there occurs a rise of agglutinins in the quarter receiving the antigen varying in degree with the animal and in rapidity according to former treatment with the same antigen. This rise is accompanied by an increase of specific blood agglutinins usually higher than that of the treated quarter. In fact injection into an udder duct is as effective as subcutaneous or intravenous injection in causing agglutinins to appear in the blood. The rise in agglutinins in the treated quarter may be associated with a slight rise in one or more of the remaining quarters.

If the first injection is followed by a second into another quarter 3 or more weeks later the rise in this quarter is more rapid and more pronounced than that following the first injection. Moreover, there is a simultaneous jump of the agglutinin titer in the quarter which was treated first and there may be a slight additional rise in the two remaining quarters.

These facts are summarized in Table VIII. Only the limiting agglutinin values are given there. The first line of figures under each case represents the maximum titer of each quarter after the first injec-

TABLE VIII.

Summary of Udder Reactions Giving Maximum Output of Agglutinins in the Blood and Milk after Each Injection.

R. F.	R. H.	L. F.	L. H.	Blood.	Daily milk secretion.
					lbs.
Eastern Cow 523. Injection of living bacilli into right fore quarter and left fore quarter.					9
1:640	1:320−	1:320−	1:320−	1:1,280	
1:640	1:320−	**1:640**	1:320	1:2,560	
Native Cow 487. Subcutaneous and intravenous injections of dead bacilli.					8
1:40	1:20	1:20+	1:40	1:2,560	
Injection into right fore quarter.					
1:640	1:320	1:320	1:160	1:2,560	
Eastern Cow 584. Injection of dead bacilli into right fore quarter and right hind quarter.					18
1:320	1:40	1:80+	1:80+	1:640	
1:640	**1:640**	1:40	1:40	1:640	
Western Cow 600. Injection of dead bacilli into right fore quarter and left fore quarter.					25
1:40	1:20	1:20	1:20	1:640	
1:160	1:40	**1:320**	1:80	1:1,280	
Western Cow 601. Injection of dead bacilli into right fore quarter and right hind quarter.					25
1:20	1:10−	1:10−	1:10−	1:320	
1:40	**1:40**−	1:10	1:20−	1:160	
Western Cow 897. Repeated injection of dead bacilli into right fore quarter.					30
1:20	1:10	1:10	1:10−	1:320	
1:160	1:20	1:20	1:20	1:320	
1:320	1:20	1:20+	1:20−	1:320	

tion, or, in the second case, after preliminary injection into subcutis and vein. The second line represents the maximum titer after the second treatment. The bold faced figure represents the titer of the treated quarter. The column next to the last contains the maximum blood titer of the period, the last the daily output of milk at the beginning of the experiment. The milk titer before treatment was either 1:10 or less. Dilutions under 1:10 were not tested.

In evaluating these figures it must be borne in mind that the udders of the experimental cows were in a state of active secretion. The product was not a stored product as is the case with colostrum, in which antibodies tend to accumulate and rapidly disappear when the secretory activity is fully established. There is here a steady loss of agglutinins which is daily replaced over considerable periods of time. This daily loss differed from cow to cow, as shown in the last column of Table VIII. The two first (Nos. 523 and 487) were nearing the end of the lactation period. The others were probably at or near the maximum of productivity.

Of interest as a collateral phenomenon is the "inflammatory" reaction immediately following the injection of dead bacteria into an udder duct. A slight febrile disturbance appears and lasts about 24 hours. The injected quarter becomes swollen, tender, and feels warmer than normal. There is an abundant outpouring of polynuclear leucocytes forming a deposit up to 8 per cent of the total fluid secreted. This reaction is chronologically independent of the rise in agglutinins. Following the first injection the latter appeared fully a week later. Tentatively we may assume the presence of a pyrogenic and an agglutinogenic factor in the killed bacilli.

The material results of the experiments are too meager to warrant any but cautious and tentative interpretations pending further investigation.

The evidence as far as it goes appears to support the hypothesis that after the injection of living or dead bacteria into the udder ducts the increased agglutinin is produced mainly in the udder tissue and that it is not due to an increased permeability of the endothelium or the epithelium of the gland. The increase is localized in the injected quarter and the one previously injected. In Experiment I the general increase in all quarters is probably due to the diffusion of living bacteria into the other quarters. In Experiment II there is

a decided increase in the quarters not injected. The results differ from subsequent ones in this respect. Two explanations suggest themselves. One is the previous treatment of the animal by the subcutaneous and intravenous route. The other is the possibility that some bacilli escaped destruction in the heating of the suspension to be injected. A contributory factor is the low daily output of milk as compared with that of the other cows.

There seems to be no valid reason to bring the rise and persistence of agglutinins in the treated quarter into relation with the prompt high output of polynuclear leucocytes. This appears within 20 hours whereas the rise in agglutinins comes on gradually and is definitely at its maximum in 10 or more days. The tendency of the cellular emigration is rather to sweep out the injected bacteria and thus reduce quantitatively their effectiveness as antigens.

The intimate physical association between udder and blood is demonstrated in several ways in the experiments. The injection into the cistern where the various ducts meet to form the single excretory duct of a suspension of dead bacilli is followed within 20 hours by a febrile reaction and a heavy emigration of polymorphonuclear leucocytes. The injected fluid in no instance was more than 20 cc. and it would hardly be expected to come in contact with the secretory epithelium. The reaction, however, is pronounced.

Another fact which shows the interchange between the blood and the udder is the prompt general response of the body to the antigens by the appearance of agglutinins in the blood in a concentration usually higher than that developed in the treated quarter. This interchange which is very prompt must be carried on through the secreting epithelium of the gland. The injection into the udder ducts is equivalent in its effects to an injection into the trachea, or even into the circulation. These facts indicate an active inward absorptive current probably alternating with active secretion.

CONCLUSIONS.

The foregoing experimental cases point to a distinct participation of the udder in the production of agglutinins when the gland is invaded by living or flooded by dead bacteria. The quarter injected reacts at first with a heavy influx of polynuclear leucocytes and later with an increase of agglutinins.