STUDIES IN THE RELATION OF MUCIN TO THE VIRULENCE OF PNEUMOCOCCI

by

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INTRODUCTION

Historical

Bacteria highly pathogenic for man sometimes present a problem for laboratory study because they may be of very low virulence for the experimental animals available. This difficulty is experienced especially in immunological studies, and particularly in the problem of titrating antimeningococcus serum. It was this problem that excited various investigators to continue Nungester's [1] experiments which showed that gastric mucin greatly enhanced the virulences of Pneumococcus type II, a hemolytic Streptococcus, and Staphylococcus aureus in mice. Miller perfected the technique, and found that a maximum virulence of meningococci for mice was obtained with peritoneal exudate diluted with mucin for inoculum.[2] Subsequently he found it possible to test the effect of antibacterial immune sera in this convenient laboratory animal, using 0.5 c.c. of dilutions of the anti-serum one-half hour before inoculation of a 1 c.c. suspension of meningococci in mucin.[3] Continuing in this field, Rake[4] found mucin, suspended by Miller's second method,[5] to increase the virulence

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1. Nungester, W. J., Wolfe, A. A. and Jourdonais, L. F. Proc. Soc. Exp. Biol. and Med.: 30: 120. 1932.
2. Miller, C. Phillip. Science: 78: 340. 1933.
3. Miller, C. P. Proc. Soc. Exp. Biol. and Med.: 32: 1140. 1935.
4. Rake, Geoffrey. Proc. Soc. Exp. Biol. and Med.: 32: 1523; 1175. 1935. J. Exp. Med.: 61: 545. 1935.
5. Miller, C.P. Proc. Soc. Exp. Biol. and Med.: 32: 1136. 1935.

of meningococci 100,000 to 1,000,000 times, and by standardizing his inocula with a Gates turbidometer, offered this method as a means of titrating antimeningococcal serum. More recently, Mishulow and Melman[6] have standardized such serum using mice and Miller's second method of suspending mucin.

Interest in this phenomenon itself has taken shape in experimental studies on other bacterial organisms and other substances, mucins or otherwise. Ruth Henderson says, "It has been found possible by the mucin technique to increase the virulence of meningococci and typhoid bacillus for mice roughly a million times in comparison with previous tests."[7] Powell and Jamieson[8] found that although mucin does not enhance the virulence of H. pertussis, starch is successful in doing so. Thus it is evident that here is a reaction, of some interest and practical importance, worthy of further investigation.

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6. Mishulow, L. and Melman, M. J. Lab. and Clin. Med.: 21: 406. 1936.
7. Henderson, Ruth, Jamieson, W. A. and Powell, H. M. Proc. Ind. Acad. Sci.: 45: 133-138. 1936.
8. Powell, H. M. and Jamieson, W. A. Proc. Soc. Exp. Biol. and Med.: 34: 435. 1936.

Mechanism of the Reaction

Actually, the mechanism of the effect of mucin on bacteria is still unknown, and we have only a few indications which can be pointed out. The general opinion at present is that the mucin exerts a protective action on the organisms.

It is known that mucin itself is non-toxic for mice in doses of 2 c.c.,[9] and that the reaction does not occur because of increased susceptibility of the animals due to irritation of the peritoneum. Other means of irritating the peritoneum, and blocking of the fixed phagocytes with India ink did not hasten the invasion of bacteria; however, injection of mucin five hours previous to the bacterial injection still produces the same increase of virulence.[10]

Several facts indicate that the mechanism might be one of a protective coating. Agar, starch and gelatin (viscous substances which are of such a physical nature as to make possible such a coating) also tend to increase the virulence of organisms, although not nearly so effectively.[11] This would point away from any idea of a specific reaction between mucin and organisms, and toward a more general physical phenomenon. The growth of bacteria on media containing mucin produces no increase in virulence,[12] which tends to exclude as a cause an actual change in the bodies of the organisms.

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9. Miller, C. P. Science: 78: 340. 1933.
10. Nungester, Wolfe and Jourdonais, op. cit.
11. Ibid.
12. Rake, G. Proc. Soc. Exp. Biol. and Med.: 32: 1523. 1935.

The close relationship of phagocytosis to virulence points to several possibilities. It is known that a virulent strain is phagocyted to a much smaller extent than a less virulent strain of organisms, regardless of the numbers injected.[13] Thus, any substance which could cause a resistance to the polymorphonuclear leukocytes (which constitute two-thirds of the cells found in the peritoneum after four hours[14])by forming, for example, a coating around the bacteria, would considerably enhance virulence. However, this also points in the opposite direction, because there is no experimental evidence to show that the mucin does not act by inhibiting the phagocytes--by coating them, perhaps, or by a toxic effect.

"Not all batches of mucin obtained from commercial sources are alike in their power of enhancing the pathogenic activity of meningococcus strains."[15] Miller himself stated that the only gastric mucin which was successful was "Granular Mucin" of the Wilson Laboratories, Chicago;[16] it was this preparation that he used in his second method of suspending mucin, and which probably most of the workers who have followed his method have used.

This peculiarity is still quite in keeping with the theory of a protective action, although these differences in action

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13. Miller, C. P. Proc. Soc. Exp. Biol. and Med.: 32: 1138. 1935.
14. Ibid.
15. Rake, G. J. Exp. Med.: 61: 545. 1935.
16. Miller, C. P. Proc. Soc. Exp. Biol. and Med.: 32: 1136. 1935.

of different kinds of mucin cannot at present be explained on a chemical basis, partly because the chemistry of mucins is as yet in a rather confused state. Briefly, mucins are glycoproteins, which, in turn are made up of a protein molecule plus a substance containing a carbohydrate group other than nucleic acid.[17] The non-protein radical is a conjugated sulfuric acid: sulfuric acid plus acetic acid plus glucuronic acid plus a hexosamine which determines whether it is mucoitin- or chondroitin-sulfuric acid.[18] "Thus, for mucoproteins . . . . . the chief variable component is not the carbohydrate complex but the protein group."[19] In this way a difference in protein groups obtained from various sources might explain a difference in action; and it would also explain the difference obtained with the same mucin (gastric) prepared in different ways.

Levene also states, in discussing the biological significance of mucoproteins, that its function in secretions is mainly protective.[20]

A few facts have been reported, however, which do not tie up very well with the simple, protective idea for the action of mucin. It has been mentioned before that Powell and Jamieson found starch and not mucin to enhance the virulence of H.

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17. Bodansky, Meyer. Introduction to Physiological Chemistry. N.Y. p. 65. 1934.
18. Levene, P. A. Hexosamines and Mucoproteins. N. Y. 1925. p. 74.
19. Ibid.
20. Ibid.

pertussis.[21] One would be inclined to think that the properties causing a substance to enhance virulence most in one organism, would also hold for another, but the great variation between organisms, one producing a deadly toxin, another growing anaerobically, or being very resistant, another filling the blood stream by its multiplication, is undoubtedly a factor here. Another peculiar thing is that the site of injection seems to make a great difference: injection in the tail vein, and under the skin does not show increased virulence in mucin-suspended organisms, as does the intra-peritoneal inoculation.[22]

It is evident, then, that this phase of the phenomenon is wide-open to an inquisitive student, and to one who has, perhaps an immediate, practical problem before him.

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21. Powell and Jamieson, op. cit.
22. Nungester, Wolfe, and Jourdonais, op. cit.

EXPERIMENTAL

Purpose

The immediate aims of these studies were: (1) to confirm the reports of mucin enhanced virulence of other workers, (2) to extend experimental data over other mucins, and (3) to touch upon the mechanism of the phenomenon, especially in the effect of mucin upon the growth of organisms.

I. Experiments on the Virulence-enhancing Effect of Mucins

Procedure

The experimental animals were mice.

A pneumococcus of Type I was used. For all work, twenty-four hour cultures of this organism were grown in beef infusion, glucose, carbonate broth. Its virulence was quite low for mice: 1 c.c. of a twenty-four hour culture killed the animal in two to three days, while a 1:10 dilution killed in three to four days.

	Dilution	3 days after injection
Mouse # 1	1:10	Dead
Mouse # 2	1:100	Survived
Mouse # 3	1:1000	Survived

This organism seemed, however, to be rather well capsulated.

Three mucins were used, granular gastric mucin (Type 1701-W) from the Wilson laboratories, powdered gastric mucin prepared by Klein, and a snail mucin (Burroughs Wellcome and Co., New York); gum acacia was employed as a viscous control. All of these substances were suspended by Miller's second method, which is quoted with his first method also for comparison:

> Miller's First Method: "Hog's gastric mucin is washed for several days in many changes of 70% alcohol to remove unnecessary ingredients and also to kill the spore of contaminating bacteria. It is then dried between blotting-paper and dissolved in sufficient saline to make a 6% solution and buffered at pH 7.4 as is ordinary culture media. Solution is facilitated by adding the saline slowly to the mucin as it is being ground vigorously in a mortar. The solution is then tubed and sterilized in an Arnold sterilizer at 100° C. for one hour every other day for three days, and incubated on the intervening days. The precipitate which forms is discarded and only the clear supernatant used for injection."[23]

> Miller's Second Method: "A 5% suspension is made up as follows: Onto the weighed quantity of mucin is poured enough distilled water to moisten it thoroughly. It is allowed to stand ½ hour. Then the sticky mass is stirred and rubbed free of solid lumps. Stirring continues as distilled water is slowly added until the final concentration of 5% is reached. The reaction of the suspension should be about pH = 5. It is placed in suitable containers and autoclaved at 15 lbs. pressure for 15 minutes. After cooling, the reaction is adjusted with normal NaOH to pH = 7.3 and glucose added to a concentration of 1%. After incubating one day to insure sterility, the suspension is ready for use. As the product now being used contains particles of sufficient size to occlude fine hypodermic needles, it has been found convenient to withdraw the supernatant from this course sediment which rapidly settles to the bottom of the container and is

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23. Miller, C. P. Science: 78: 340. 1933.

> easily visible to the naked eye. The supernatant, hereinafter designated the "mucin suspension", tends itself to settle out on standing, particularly in the cold, should be thoroughly mixed by gentle agitation before using, in order to insure its uniform composition in all experiments."[24]

In these preparations it was found necessary to follow the directions closely, firstly, in order to produce a satisfactory product, and secondly, in order to obtain results, confirmatory or otherwise, correlative with those of other workers. A short-cut in this preparation attempted by way of adjusting the pH before autoclaving was a complete failure because the suspension came out of the autoclave very dark-colored and semi-transparent, evidently unsuitable for use. After sterilizing, then, the pH was adjusted with color standards of methyl orange indicator. For 100 c.c. of the suspension, approximately the following amounts of base or acid were needed to obtain a pH of 7.3:

Granulated gastric mucin - - 1.3 c.c. of N NaOH

Powdered gastric mucin - - - 1.2 c.c. of N NaOH

Snail mucin - - - - - - - - - 0.5 c.c. of N HCl

Although the suspensions tend to settle out more rapidly in the cold it was found advisable to keep these preparations in the ice-box to prevent the growth of contaminants. Frequently they were plated to insure sterility. When they became contaminated, they could not be sterilized again, as

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24. Miller, C. P. Proc. Soc. Exp. Biol. and Med.: 32: 1136. 1935.

stated above, but new suspensions had to be prepared.

Dilutions of the twenty-four hour culture of Pneumococcus I in mucin were set up and injected intraperitoneally into mice as soon as possible. Most of the experiments were repeated to obtain definite end-points.

A time limit of three days was placed on each experiment. Any mice dying within this time were autopsied and smears and platings made of the peritoneal exudate and heart's blood to definitely establish the pneumococcus as the cause of death. In this connection I should like to make a note of the fact that although I was unable to recover the pneumococcus in plates in some of the earlier autopsies, they were undoubtedly there, because many of the smears showed large numbers of them; the failure to recover these was obviously due to poor technique, but does not necessarily invalidate the results as long as the smears were positive for Pneumococcus I.

All operations were carried out with aseptic technique.

Data

Granulated gastric mucin was found to enhance the virulence of the Pneumococcus so that a dilution of 1:1,000,000 usually killed within three days. The saline controls, however, never caused death in this length of time in a dilution above 1:10. In the following tables, the number of the animal is given so that its history may be consulted; the time limit was three days; and asterisks (*) indicate recovery of the pneumococcus from the animal, one meaning in smears only,

two, also in culture.

Table I

Dilution	Mouse	Saline	Mouse	Gran. gast. Mucin
Undiluted	# 4	Died *		
1:10	# 5	Survived	# 8	Died **
1:100	# 6	Survived	# 9	Died *
1:1000	# 7	Survived	# 10	Died *
1:10,000			# 11	Died *

Table II

Dilution	Mouse	Saline	Mouse	Gran. gast. Mucin
1:10	# 2	Died *	# 19	Died **
1:100	# 14	Survived	# 20	Died **
1:1000	# 18	Survived	# 21	Died [1]

[1]This mouse did not die of a pneumococcic septicaemia. It died much sooner than did the others, and on autopsy, showed acute hemorrhage into the peritoneal cavity.

Table III

Dilution	Mouse	Saline	Mouse	Gran. gast. Mucin
1:10,000	# 12	Survived	# 15	Survived
1:100,000	# 13	Survived	# 16	Survived
1:1,000,000	# 14	Survived	# 17	Survived

The results in this series may be accounted for on a basis of experimental error, for example, a variation in growth of the pneumococcus I in the media provided. Further experiments all show death of the animals injected with mucin suspension of the pneumococcus.

Table IV

Dilution	Mouse	Saline	Mouse	Gran. gast. Mucin
1:1,000	# 22	Survived	# 25	Died **
1:10,000	# 23	Survived	# 26	Died
1:100,000	# 24	Survived	# 27	Died *

Table V

Dilution	Mouse	Gran. gast. Mucin	Mouse	Gran. gast. Mucin
1:1,000	# 31	Died **	# 32	Survived
1:10,000	# 33	Died **	# 34	Died **
1:100,000	# 35	Died **	# 36	Died *
1:1,000,000	# 37	Died **	# 38	Died **

Table VI

Dilution	Mouse	Gran. gast. Mucin
1:1,000,000	# 42	Survived
1:10,000,000	# 43	Survived
1:100,000,000	# 44	Survived

Powdered gastric mucin and snail mucin were found to be about equal in their effect on the virulence of the pneumococcus; they both increased its virulence, although not nearly so much as did the granular gastric mucin. Both of these mucins enabled the pneumococci to kill the mice consistently in a dilution of 1:1000, and often in a dilution of 1:10,000. The following tables show the results of repeated experiments:

Table VII

Dilution	Mouse	Snail Mucin	Mouse	Pwd. gast. Mucin
1:10	# 53	Died **	# 56	Died **
1:100	# 54	Died **	# 57	Died **
1:1000	# 55	Died *	# 58	Died **

Table VIII

Dilution	Mouse	Snail Mucin	Mouse	Pwd. gast. Mucin
1:100	# 59	Died **	# 62	Died **
1:1000	# 60	Died **	# 63	Died **
1:10,000	# 61	Died **	# 64	Died **

Table IX

Dilution	Mouse	Snail Mucin	Mouse	Pwd. gast. Mucin
1:10,000	# 65	Survived	# 68	Survived
1:100,000	# 66	Survived	# 69	Survived
1:1,000,000	# 67	Survived	# 70	Survived

Gum acacia was also tried for its effect on the virulence of pneumococcus I, and was found to have no effect whatever:

Table X

Dilution	Mouse	Gum acacia
1:10	# 45	Survived
1:100	# 46	Survived
1:1000	# 47	Survived
1:10,000	# 48	Survived

A skin test on rabbits, corresponding to this work was performed by H. V. Ellingson of the department of Medical Bacteriology here. In the same suspension of granulated gastric mucin which I used, he suspended varying dilutions of a pneumococcus I, and injected 0.1 c.c. subcutaneously. He obtained results confirming mine: the mucin greatly enhanced the virulence of the pneumococcus. His results were as follows:

Dilution	Reaction in Saline Suspension	Reaction in Mucin Suspension
1:1000	Very slight skin reaction.	Erythema and oedema about 50 mm. in diameter.
1:10,000	Negative.	Erythema and oedema about 25 mm. in diameter.

II. Experiments on the Growth-inhibiting Effect of Mucins

Procedure

A twenty-four hour culture of the organism was diluted by putting one drop in five c.c. of broth. One drop of this was added to tubes containing two c.c. amounts of varying dilutions of mucin in broth. The number of organisms present in these mixtures was indicated by plating one 3 mm.-loopful in poured agar plates. These tubes were then incubated for four hours, at the end of which time plates were made again in the same way. The two sets of plates were compared after twenty-four hours incubation, to determine the effect of the mucin on the growth.

The organisms used were B. coli, Bacillus mucosus capsulatus, Park 8 strain of the diphtheria bacillus, and Pneumococcus I.

Data

Granulated gastric mucin caused slight inhibition of the growth of all of the bacteria used, except that of the pneumococcus, in which case inhibition of growth was complete in the high concentrations (a fact which may have been a matter of time for the slower-growing pneumococcus).

In the following tables, the first plating was set up as a control, and arbitrarily read as "+" (these plates usually

contained approximately 200 colonies). The later platings were compared with this, and the increase in colonies being most high in the broth control, that plate was called "++++".

Powdered gastric mucin showed the same property of slightly inhibiting growth, most markedly for the pneumococcus, and quite markedly for bacillus mucosus capsulatus. (Table XII)

Snail mucin also slightly inhibited growth, especially with the pneumococcus. (Table XIII)

Table XI

Granular Gastric Mucin

Mucin Dilution	B. coli	B.M.C.	Park 8	Pneumo.
	Plated immediately			
100%	+	+	+	+
50%	+	+	+	+
25%	+	+	+	+
10%	+	+	+	+
Control	+	+	+	+
25%				+
10%				+
5%				+
2.5%				+
1%				+
Control				+
	Plated after 4 hours			
100%	+++	++	+	±
50%	++++	+++	++	-
25%	++++	++++	++	-
10%	++++	++++	++	±
Control	++++	++++	++	+
25%				-
10%				+
5%				+
2.5%				+
1%				+
Control				+

Table XII

Powdered Gastric Mucin

Mucin Dilution	B. coli	B.M.C.	Park 8	Pneumo.
	Plated immediately			
100%	+	+	+	+
50%	+	+	+	+
25%	+	+	+	+
10%	+	+	+	+
Control	+	+	+	+
25%				+
10%				+
5%				+
2.5%				+
1%				+
Control				+
	Plated after 4 hours			
100%	+++	+	+	-
50%	++++	+++	++	-
25%	++++	++++	++	-
10%	++++	++++	++	-
Control	++++	++++	++	+
25%				+
10%				+
5%				+
2.5%				+
1%				+
Control				+

Table XIII

Snail Mucin

Mucin Dilution	B. coli	B.M.C.	Park 8	Pneumo.
Plated immediately				
100%	+	+	+	+
50%	+	+	+	+
25%	+	+	+	+
10%	+	+	+	+
Control	+	+	+	+
25%				+
10%				+
5%				+
2.5%				+
1%				+
Control				+
Plated after 4 hours				
100%	++	+++	+	+
50%	+++	++++	++	-
25%	++++	++++	++	+
10%	++++	++++	++	+
Control	++++	++++	++	+
25%				+
10%				+
5%				+
2.5%				+
1%				+
Control				+

CONCLUSIONS

This work has shown that granular gastric mucin enhances the virulence of Pneumococcus I approximately 100,000 times, which confirms the reports of the others who have conducted such experiments.

Further, it was found that snail mucin and powdered gastric mucin also enhanced the virulence of the same organism, but not as effectively as did the other. Why there should be such a difference in this power between substances so similar, still remains to be determined. It may be due to a difference in physical properties which causes a corresponding difference in the abilities of the several kinds to coat bacterial bodies, or to one which causes different degrees of inhibition of the phagocytes.

It might be deduced from the fact that gum acacia, which is a carbohydrate related to the sugars, had no effect on the pneumococcus, that this reaction may not be dependent on chemical constitution, because starch, which is a polysaccharide also, has been found quite effective. And it must be remembered that a five per cent solution of starch is considerably more viscous than such a one of gum acacia. This physical difference in the two tends to lend importance to the physical properties of these virulence-enhancing substances.

From the results of the experiments on the growth inhibiting effect of mucin, it would seem that the mechanism by which

the virulence is enhanced is not one which stimulates the bacteria to more rapid growth within the animal, because in higher concentrations there is even a slight inhibition of such growth. The fact that the pneumococcus is more inhibited than are the others is not a striking one in the light of its delicacy in artificial media. Since it needs special media to begin with, such a change as adding twenty-five per cent mucin may easily disturb its life functions. Because it is somewhat slower to grow, perhaps four hours was not sufficient to produce good results.

Summary

1. Granular gastric mucin enhances the virulence of Pneumococcus I approximately 100,000 times.

2. Snail mucin and powdered gastric mucin enhance the virulence of the same organism about 1000 times.

3. Gum acacia has no effect on the virulence of this organism.

4. These mucins slightly inhibit the growth of various bacteria.

RECORDS

OF THE EXPERIMENTAL MICE

 # 1 Injected with 1:10 dilution in saline, February 20.

Died within 60 hours.

No autopsy.

 # 2 Injected with 1:100 dilution in saline, February 20.

Survived.

Injected with 1:10 dilution in saline, March 17.

Died within 3 days, March 20.

Autopsy: very little peritoneal exudate obtained.

Peritoneal exudate	Heart's blood
Smear shows Gram-positive diplococci.	Smear shows very few Gram-positive diplococci.
Plate is almost sterile; negative Pneumococcus.	Plate is almost sterile; negative Pneumococcus.

 # 3 Injected with 1:1000 dilution in saline, February 20.

Survived.

 # 4 Injected with undiluted broth culture, February 23.

Died February 26, within 60 hours after injection.

Autopsy: Very little blood in heart, little exudate; the animal appeared emaciated.

Peritoneal exudate	Heart's blood
Smear shows diplococci present, and other organisms.	Smear shows a few diplococci.

5 Injected with 1 c.c. 1:10 dilution in saline, February 23.

Died after 4 days.

No autopsy.

6 Injected with 1:100 dilution in saline, February 23.

Died after 4 days.

No autopsy.

7 Injected with 1:1000 dilution in saline, February 23.

Died after 7 days, March 3.

Autopsy:

Peritoneal exudate

Smears show many Gram-pos. diplococci and some leukocytes.

Plates were completely contaminated.

8 Injected with 1:10 dilution in granular gastric mucin, February 23.

Died within 18 hours, February 24.

Autopsy: a good quantity of peritoneal exudate was obtained.

Peritoneal exudate	Heart's blood
Smear shows enormous numbers of pneumococci; also other organisms.	Smear shows pure pneumococci present.
Plates show pneumococci, with contaminants.	Plate shows only contamination.

9 Injected with 1:100 dilution in granular gastric mucin, February 23.

Died within 20 hours, February 24.

Autopsy: small quantity of peritoneal exudate was obtained.

Peritoneal exudate	Heart's blood
Smear shows pure penumococci.	Smear shows pure pneumococci.
Plates sterile.	Plates sterile.

10 Injected with 1:1000 dilution in granular gastric mucin, February 23.

Died within 26 hours, February 24.

Autopsy: moderate amount of peritoneal exudate obtained.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci. Some of the cocci appear very large.	Smear shows pneumococci. Some of the cocci appear very large.
Plate is almost sterile.	Plate is almost sterile.

11 Injected with 1:10,000 dilution in granular gastric mucin, February 23.

Died within 48 hours, February 24.

Autopsy: no heart's blood was obtained.

Peritoneal exudate

Smear shows many pneumococci; some parts of the smear show capsules (Gram stain).

Plate is sterile.

12 Injected with 1:10,000 dilution in saline, March 4.

Died after 5 days.

No autopsy.

13 Injected with 1:100,000 dilution in saline, March 4.

Died after a week.

No autopsy.

14 Injected with 1:1,000,000 dilution in saline, March 4.

Survived.

Injected with 1:100 dilution in saline, March 17.

Survived.

15 Injected with 1:10,000 dilution in granular gastric mucin, March 4.

Died after a week.

No autopsy.

16 Injected with 1:100,000 dilution in granular gastric mucin, March 4.

Died after a week.

No autopsy.

17 Injected with 1:1,000,000 dilution in granular gastric mucin, March 4.

Survived.

18 Injected with 1:1000 dilution in saline, March 17.

Survived.

19 Injected with 1:10 dilution in granular gastric mucin, March 17.

Died after 30 hours, March 19.

Autopsy: moderate amount of peritoneal exudate obtained.

Peritoneal exudate	Heart's blood
Smear shows enormous numbers of gram-positive diplococci.	Smear shows Gram-positive diplococci.
Plate shows pneumococci and a contaminant.	Plate shows pure culture of pneumococci.

20 Injected with 1:100 dilution in granular gastric mucin, March 17.

Died after 30 hours, March 19.

Autopsy: Very little peritoneal exudate present.

Peritoneal exudate	Heart's blood
Smear shows Gram-positive diplococci.	Smear shows Gram-positive diplococci.
Plate shows pure culture of pneumococci.	Plate shows almost pure culture of pneumococci.

21 Injected with 1:1000 dilution in granular gastric mucin, March 17.

Died before 24 hours, March 18.

Autopsy: much hemorrhage in the peritoneal cavity. All exudate wass bloody. This animal bled on injection, at site of injection.

Peritoneal exudate	Heart's blood
Smear shows a few Gram-positive diplococci, also Gram-positive rods, and other organisms, r.b.c. and leukocytes were seen.	Smear shows no pneumococci.
Plate was contaminated, and had no pneumococci.	Plate was almost sterile.

22 Injected with 1:1,000 dilution in saline, March 28.

Survived.

Injected with 1:1,000 dilution in mucin, April 2.

Survived.

23 Injected with 1:10,000 dilution in saline, March 28.

Survived.

Injected with 1:10,000 dilution in granular gastric mucin, April 2.

Died within 20 hours, April 3.

Autopsy: much peritoneal exudate obtained, and 1 drop of heart's blood.

Peritoneal exudate	Heart's blood
Smear shown no pneumococci; cocci were present.	Smear shows no pneumococci; cocci were present.
Plate shows contamination only.	Plate has contamination only.

24 Injected with 1:100,000 dilution in saline, March 28.

Survived.

Injected with 1:100,000 dilution in granular gastric mucin, April 2.

Died within 20 hours, April 3.

Autopsy: 2 drops of peritoneal exudate obtained, and 3 drops of heart's blood.

Peritoneal exudate	Heart's blood
Smear shows some Gram-positive cocci only.	Smear shows no organisms.
Plate had 2 colonies of pneumococci.	Plate was completely contaminated.

25 Injected with 1:1,000 dilution in granular gastric mucin, March 28.

Died within 45 hours, March 30.

Autopsy: Animal had been dead for some time.

Peritoneal exudate	Heart's blood
Smear shows large numbers of pneumococci.	
Plate was completely contaminated.	Plate had pneumococci present.

26 Injected with 1:10,000 dilution in granular gastric mucin, March 28.

Died within 20 hours, March 29.

Autopsy: Much peritoneal exudate; good quantity of heart's blood; small hemorrhage at site of injection on body wall.

Peritoneal exudate	Heart's blood
Smear shows Gram-positive diplococci and other organisms.	Smear shows no pneumococci.
Plate was completely contaminated.	Plate was completely contaminated.

27 Injected with 1:100,000 dilution in granular gastric mucin, March 28.

Died within 45 hours, March 30.

Autopsy: dead some time, partially decomposed.

Peritoneal exudate	Heart's blood
Smear shows large numbers of Gram-positive diplococci.	Smear shows large numbers of Gram-positive diplococci.
Plate shows no pneumococci; much contamination.	Plate shows no pneumococci; much contamination.

28 Injected with 1:1,000 dilution in saline, April 2.

Survived.

29 Injected with 1:10,000 dilution in saline, April 2.

Died after a week.

No autopsy.

30 Injected with 1:100,000 dilution in saline, April 2.

Survived.

31 Injected with 1:1,000 dilution in granular gastric mucin, April 13.

Died within 40 hours, April 15.

Autopsy: several drops of heart's blood and peritoneal exudate obtained.

Peritoneal exudate	Heart's blood
Smear shows many Gram-positive diplococci.	Smear shows many Gram-positive diplococci.
Plate has pure culture of pneumococci.	Plate is sterile.

32 Injected with 1:1,000 dilution in granular gastric mucin, April 13.

Survived.

33 Injected with 1:10,000 dilution in granular gastric mucin, April 13.

Died within 65 hours, April 16.

Autopsy: small amount of peritoneal exudate and very little heart's blood were obtained.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows pneumococci.
Plate has pure culture of pneumococci.	Plate is sterile.

34 Injected with 1:10,000 dilution in granular gastric mucin, April 13.

Died within 65 hours, April 16.

Autopsy: large amount of dark green peritoneal exudate was present, and the heart's blood was in lumps.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows pneumococci.
Plate has pure culture of pneumococci.	Plate has pure culture of pneumococci.

35 Injected with 1:100,000 dilution in granular gastric mucin, April 13.

Died after 48 hours, April 14.

Autopsy: peritoneal exudate and heart's blood were obtained.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci present.	Smear shows pneumococci.
Plate has pure culture of pneumococci.	Plate was sterile.

36 Injected with 1:100,000 dilution in granular gastric mucin, April 13.

Died within 72 hours, April 16.

Autopsy: peritoneal exudate and very little heart's blood was obtained.

Peritoneal exudate	Heart's blood
Smear shows pneumococci.	Smear shows a few pneumococci, and some Gram-negative rods.
Plate, negative	Plate, negative.

37 Injected with 1:1,000,000 dilution in granular gastric mucin, April 13.

Died after 48 hours, April 14.

Autopsy: peritoneal exudate and heart's blood was obtained.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows pneumococci.
Plate had pneumococci and a contaminant.	Plate was sterile.

38 Injected with 1:1,000,000 dilution in granular gastric mucin, April 13.

Died within 72 hours, April 16.

Autopsy: peritoneal exudate and very little heart's blood were obtained.

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows very few pneumococci.
Plate had pneumococci and contamination.	Plate showed a few colonies of pneumococci in pure culture.

39 Injected with 1:10 dilution in snail mucin, April 18.

Died after 24 hours, April 19.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows only Gram-negative rods.	Smear shows no organisms.
Plate, negative pneumococcus.	Plate, negative pneumococcus.

40 Injected with 1:100 in snail mucin, April 18.

Survived.

41 Injected with 1:1000 in snail mucin, April 18.

Died after a week, April 25.

No autopsy.

42 Injection with 1:1,000,000 dilution in granular gastric mucin, April 18.

Survived.

43 Injected with 1:10,000,000 dilution in granular gastric mucin, April 18.

Survived.

44 Injected with 1:100,000,000 dilution in granular gastric mucin, April 18.

Survived.

45 Injected with 1:10 dilution in gum acacia, April 20.

Died after 4 days.

No autopsy.

46 Injected with 1:100 dilution in gum acacia, April 20.

Survived.

47 Injected with 1:1000 dilution in gum acacia, April 20.

Survived.

48 Injected with 1:10,000 dilution in gum acacia, April 20.

Survived.

49 Injected with 1:1,000 dilution in snail mucin, April 20.

Survived.

50 Injected with 1:10,000 dilution in snail mucin, April 20.

Survived.

51 Injected with 1:100,000 dilution in snail mucin, April 20.

Died within 30 hours, April 21.

Autopsy: (dead over-night)

Peritoneal exudate	Heart's blood
Smear shows no organisms.	Smear shows no organisms.
Plate was almost sterile, negative pneumococcus.	Plate was s terile.

52 Injected with 1:1,000,000 dilution in snail mucin, April 20.

Died within 48 hours, April 21.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows no organisms.	Smear shows one pneumococcus.
Plate was sterile.	Plate was sterile.

53 Injected with 1:10 dilution in snail mucin, April 30.

Died after 24 hours, May 1.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many Gram-positive diplococci.	Smear shows a few Gram-positive diplococci.
Plate has pneumococci present.	Plate has pure culture of pneumococci.

54 Injected with 1:100 dilution in snail mucin, April 30.

Died after 24 hours, May 1.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows pneumococci and another organism.	Smear shows a few pneumococci.
Plate completely contaminated.	Plate completely contaminated. Pneumococci present.

55 Injected with 1:1000 dilution in snail mucin, April 30.

Died within 64 hours, May 2.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows pneumococci.	Smear shows pneumococci.

Plate completely contaminated.	Plate completely contaminated.

56 Injected with 1:10 dilution in powdered gastric mucin, April 30.

Died within 44 hours, May 2.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many Gram-positive diplococci.	Smear shows pneumococci.
Plate has pneumococci.	Plate has pneumococci.

57 Injected with 1:100 dilution in powdered gastric mucin, April 30.

Died within 44 hours, May 2.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many Gram-positive diplococci, and other organisms.	Smear shows many pneumococci.
Plate was completely contaminated.	Plate had pneumococci.

58 Injected with 1:1000 dilution in powdered gastric mucin, April 30.

Died within 44 hours, May, 2.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows many pneumococci.
Plate was completely contaminated.	Plate had pneumococci.

59 Injected with 1:100 dilution in snail mucin, May 4.

Died within 72 hours, May 6.

Autopsy: pus in the peritoneal cavity.

Peritoneal exudate	Heart's blood
Smear shows pneumococci.	Smear shows a few pneumococci.
Plate was completely contaminated.	Plate had pneumococci.

60 Injected with 1:1000 dilution in snail mucin, May 4.

Died after 48 hours, May 6.

Autopsy: pus in the peritoneal cavity.

Peritoneal exudate	Heart's blood
Smear shows enormous numbers of pneumococci.	Smear shows pneumococci.
Plate has pure culture of pneumococci.	Plate has pure culture of pneumococci.

61 Injected with 1:10,000 dilution in snail mucin, May 4.

Died after 48 hours, May 6.

Autopsy: pus in the peritoneal cavity.

Peritoneal exudate	Heart's blood
Smear shows enormous numbers of pneumococci.	Smear shows pneumococci.
Plate has pure culture of pneumococci.	Plate has pure culture of pneumococci.

62 Injected with 1:100 dilution in powdered gastric mucin, May 4.

Died within 72 hours, May 6.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows pneumococci.	Smear shows pneumococci.
Plate has pneumococci.	Plate has pneumococci.

63 Injected with 1:1000 dilution in powdered gastric mucin, May 4.

Died within 24 hours, May 5.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many pneumococci and other organisms.	Smear shows Gram-positive diplococci, and other cocci.
Plate was completely contaminated.	Plate has pneumococci.

64 Injected with 1:10,000 dilution in powdered gastric mucin, May 4.

Died within 44 hours, May 6.

Autopsy:

Peritoneal exudate	Heart's blood
Smear shows many pneumococci.	Smear shows many pneumococci.
Plate has pneumococci.	Plate has pneumococci.

65 Injected with 1:10,000 dilution in snail mucin, May 19.

Died after 5 days, May 24.

No autopsy.

66 Injected with 1:100,000 dilution in snail mucin, May 19.

Survived.

67 Injected with 1:1,000,000 dilution in snail mucin, May 19.

Survived.

68 Injected with 1:10,000 dilution in powdered gastric mucin, May 19.

Survived.

69 Injected with 1:100,000 dilution in powdered gastric mucin, May 19.

Survived.

70 Injected with 1:1,000,000 dilution in powdered gastric mucin, May 19.

Survived.

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