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AUTHORITY

SMUFD D/A ltr, 4 Feb 1972

TRANSLATION NO. 714

DATE: July 68

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

[redacted] # 714
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Immunity to anthrax.

by G. Ivanovics.

Zeitschrift f. Immunitaetsforschung, 94: 436-458 (1938).

It is generally believed that active immunity to anthrax may be effectively produced only by the use of live bacteria; with regard to its formative mechanism it must therefore be considered a super-infectious immunity. The value of this concept, based as it is on numerous exact experimental observations, is only slightly affected by a few reports which support the view that immunity could be conferred, under certain conditions, by treatment with killed bacilli or their derivatives. In order to avoid misconceptions, it must be stressed here that those observations which deal with tests with "animalized" bacilli or their products (e.g. edematous fluid of infected animals) are not included here, and that only tests with killed, cultivated bacilli or their derivatives are involved.

The literature contains numerous references (1) to successful immunizational tests with killed bacilli cultured in different ways, or with extracts produced from them. A part of these test results could not be confirmed by re-examination, however; on the other hand, the immunity conferred by killed bacilli was so low that its specificity was doubtful. An increase in resistance caused by non-specific means must be included in considerations, since several examples undoubtedly support the existence of such a phenomenon. Pettersson (2), for instance, was able to immunise rabbits against a multiple lethal dose by injecting leukocytes. Pawlowsky (3) found increased resistance to anthrax in animals infected with diverse suppurative pathogens (Staphylococcus, Bacillus Friedlander, etc.)

During the past few years, Tomcsik and his co-workers have tried to immunize rabbits against anthrax with a killed vaccine. They used a heat-killed vaccine produced from strongly encapsulated bacilli, of which large amounts (½-1 slant agar culture) were injected intravenously 8-10 times. Immunization was continued until large quantities of precipitin were demonstrated in the animal's blood. The blood initially showed the precipitin corresponding to the somatic antigen of the bacilli, later also the capsular antibody (4,5).

These tests yielded the first proof that the capsule of anthrax bacilli is equipped with antigenic properties that differ from the properties of the bacillary body. The most conspicuous phenomenon is the one that allows the serum of rabbits treated in this manner to be used for the protection of mice against anthrax infection; according to Tomcsik and his colleagues, 1 cc confers positive protection against a 20 to 100-fold lethal dose (6,7). It was also demonstrated positively in the course of these tests that this protective effect of the serum is dependent only upon its content of capsular antibody, and that the somatic antibody has

no effect in this sense. The best proof for the factual connection between the passive immunity of the mouse and the capsular antibody was given in a successful attempt to neutralize the immunity of animals immunized with the serum in vivo by the injection of specific capsular substance (8). This property of the capsular hapten recalls to some extent the effect of "aggressins," although they cannot be identified with the concept of Bail's aggressins, since this effect of the hapten relates only to immune animals.

While mice can be given positive protection against anthrax infection with the serum of rabbits treated with killed, encapsulated anthrax bacilli, all attempts to immunize rabbits or guinea pigs with this serum failed (7). This divergence could not, however, be ascribed to the circumstance that these animals had received relatively fewer antibodies than the mice, owing to their heavier body weight, since even those rabbits did not prove to be completely immune against a heavy anthrax infection which had been repeatedly immunized with encapsulated bacilli and the blood of which contained large quantities of antibodies. These animals showed a different course of infection to the extent that the lighter infection was survived by the majority of them and that those animals which succumbed to the infection had lived slightly longer than the controls. The peculiarity of these phenomena therefore consists of the fact that the immunized rabbits which furnish the serum are not protected, or hardly protected against infection themselves, while mice treated with the serum of the same animals possess complete immunity.

Although the results mentioned here represent another failure when applied to the problem of active immunization with killed bacteria, its recognition is nevertheless extremely important since it has led to important experimental data on the role of the bacillary capsule in anthrax immunity, heretofore only suspected, but not proved. By the recognition and demonstration of the capsular antibody as one of the most important factors in the field of anthrax immunity, the earlier, heretofore insufficiently confirmed concept of Preisz (9), Gruber and Futaki (10), namely that the bacillary capsule has a function in anthrax immunity, regainst prominence. Although the authors listed in the past were only able to demonstrate unequivocally that a connection exists between the capsule of the anthrax bacillus and its virulence, the opinion nevertheless spread that the bacillary capsule has something to do with the development of immunity; there were even researchers (11) who asserted categorically that immunity to anthrax could be produced only with encapsulated bacilli.

Bail (12), who searched for the origin of the anthrax infection solely in the action of "aggressins," did not attribute any importance to the bacillary capsule in the question of anthrax infection and immunity, or he saw its function only in the circumstance that "animalised" bacilli, under the protection of the capsule, excrete "aggressins," which then furnish the stimulus for immunity. Bail attributed the development of anthrax immunity to the presence of anti-aggressins and did not consider that the capsular substance might be equipped with an antigen-like property.

It is understandable that this investigator underestimated the role of the capsule in immunity, for he succeeded in immunizing guinea pigs with a variant of anthrax bacilli which did not produce capsules under any condition (13). The number of Bail's tests mentioned here was very small, however, and he did not consider his tests suited for far-reaching conclusions.

In treating the question of immunity to anthrax, the varieties of the anthrax bacillus should not be neglected. As is well known, the virulent anthrax bacillus grows on an ordinary agar medium in the form of dry colonies with irregular borders and coarse surfaces, identified according to their appearance as variety "R" in the sense of Arkwright's nomenclature. Most of the bacilli forming these colonies are non-capsular or reveal a small part having weakly developed capsules. According to the latest concept, the colonies of virulent anthrax bacilli, in spite of their appearance, are not to be identified as R variants (14, 15, 16). If the virulent anthrax bacillus is not cultured on ordinary agar media, but on serum (17, 18, 13), on serum agar (19), on defibrinated horse serum (15) or on a medium containing 10-60% CO_2 (20, 21, 16), round, shiny colonies with smooth surfaces and a slimy appearance will invariably develop, and the bacilli of these colonies are for the most part equipped with well-developed capsules. The virulent anthrax bacilli thus grow in the form of dry, coarse-surfaced colonies with irregular margins under ordinary conditions of culture only because these conditions preclude encapsulation. Freshly isolated, virulent anthrax bacilli grown on ordinary agar cultures therefore correspond to variety "S" despite their appearance; this is supported at any rate by their strong virulence and their ability to form capsules under suitable cultural conditions. Aside from virulent anthrax bacilli which form capsules only under corresponding conditions, there are strains that produce copious capsules even on ordinary agar and that completely correspond to the S variant in their appearance. These strains are usually avirulent and are developed primarily by artificial attenuation. Thus the virulence of anthrax strains seems to be influenced by other factors besides encapsulation, claimed by Sterne (16) to be located in the plasma of the bacillary body.

Bail (13) was the first (1915) to point out that there are avirulent varieties of the anthrax bacillus which, on agar cultures, apparently do not differ from the colonies of genuinely virulent strains, showing a remarkable deviation when cultured in horse serum instead of on agar. In horse serum these strains do not form capsules at all, in contrast to the virulent strains. The pathogenicity of these non-capsular strains, dissociated from the virulent ones, may be attenuated to such an extent that only the use of large quantities permits the lethal infection of mice or guinea pigs. Animals infected and killed with large amounts of these bacilli show edema at the point of inoculation -- just as seen upon infection with virulent bacilli -- but the local reproduction of bacilli either does not lead to invasion at all, or produces only a low grade bacteriemia.

The observations by Bail recounted here were joined in time by numerous treatises by other authors who reported similar results. According to these reports, the dissociation of varieties that cease to form capsules from virulent strains takes place in different ways: Spontaneously (13,21), due to thermal effects (13), in oxalated horse serum (15), in a medium with a high CO_2 concentration (16). Since this variety forms dry, flat colonies with irregular edges and an uneven surface even under the most varied cultural conditions, and since the bacilli do not form capsules under any conditions, this type of anthrax bacillus may be identified as variety "R" in the opinion of Stamatin (15), Schaefer (14), Sterne (16). This concept can be confirmed in every way by our knowledge of the varieties S --> R of the bacilli, prompting us to retain these designations in the following.

The first immunizational tests (1915) mentioned above, with the R variant of the anthrax bacillus, are credited to Bail; such tests were resumed only in the last few years. The small number of tests conducted by Stamatin and Stamatin (22) led to the positive proof that rabbits may be successfully immunized against a severe anthrax infection with the R variant used by these investigators (1936); these tests were less decisive in connection with mice. Stamatin and Stamatin (23) were also able to successfully immunize rabbits with the edematous fluid of mice infected and killed with the R variant. The immune value of the R variant is properly illuminated by tests conducted by Sterne (16,24) at the same time. Sterne's numerous tests have not only succeeded in positively demonstrating the immunizational properties of the R variant, but have also shown the different difficulties which make the practical application of the vaccine questionable for the time being. Sterne's test results should be considered completely valid, since he used guinea pigs, which are particularly difficult to immunize against anthrax, and tested the animal's immunity with massive doses, thus completely negating the role of possibly non-specific factors.

In the investigations of Bail, Stamatin and Stamatin as well as Sterne described so far, the question of the behavior of the R variant in the living organism has been treated only on a morphological basis. These tests therefore do not preclude the possibility that non-capsular bacilli (in the morphological sense) may secrete an antigen, equivalent to the capsule, in a soluble state. Such a situation must, however, be reckoned with, since in the spore-forming saprophytes the anthrax-bacillary hapten (which represents the specific substance of sporulent, aerobic bacteria) is usually found to be extracellular (25). The re-examination of our problem from this point of view is not rendered superfluous by the circumstance that rabbits inoculated with killed, encapsulated bacilli and having great quantities of capsular antibodies in the blood, evidence a low defensive ability against anthrax infection and do not possess extensive immunity (7). It could be assumed that capsular antigen formed in the animal organism might be somewhat more effective than that of cultured bacilli.

In the tests described here, the question of the mechanism of anthrax immunity was re-examined in the sense of the concepts mentioned above. In addition, thorough investigations were conducted to establish whether further differences in respect to anthrax immunity existed between the mouse and the rabbit.

Methods.

1. Anthrax strains utilized: We used 5 different anthrax strains in our tests: 3 belonged to variety "S", they were virulent and formed capsules under suitable conditions (animal organism, presence of CO_2, etc.), while 2 strains did not produce capsules under any conditions and could be considered practically avirulent (variety "R").

Virulent strain "A_{22}" (hereafter called "A22"): This was an older strain from our collection, the virulence of which had remained unchanged for mice. We therefore used this strain primarily for the infection of mice. The virulence of the 24-hour agar culture (pH of the agar 7.3-7.5) was established 12 times between February 1936 and July 1938. In the course of these tests 31 mice were infected with 1/1,000,000 slant agar culture, of which 30 died on the 3rd-10th day and only one survived. The lethal infection was less regular when smaller quantities of this strain were used: Of 26 mice inoculated with 1/10,000,000 slant agar culture, 8 survived, and of 3 inoculated with 1/100,000,000, 2 survived. The "animalised" bacilli of this strain showed a somewhat stronger effect: Of 3 mice inoculated with 23 germs from edematous fluid, only one lived.

Virulent strain A_{33} (hereafter called "A33"): This strain was grown in the summer of 1936 from a human pustula maligna. Details on the virulence of this strain for rabbits are given in the experimental part.

Moderately virulent strain A_{15}: Although this strain was capable of forming capsules both in the mouse organism and 20% CO_2-containing blood agar culture, it nevertheless possessed only moderate virulence. The details of its virulence for mice are discussed in one of our earlier papers (7).

Avirulent strain "1190 R": This one was made available to us by N. Stamatin in February 1938. For details about its properties see (15, 22, 23).

The following may be said about our own observation of the latter strain, agreeing completely with those of Stamatin: This strain does not form capsules either on agar or on rabbit blood agar, a medium containing 25% CO_2. On 11 March 1938 we infected 6 young guinea pigs (weighing 200-300 g) with 1/50 slant agar culture subcutaneously. After 2 days a low grade edema formed at the site of inoculation, disappearing thereafter in 4 cases, but increasing in 2 animals; these 2 died on the 5th and 6th day, respectively. Dissection revealed extraordinarily severe subcutaneous edema, containing copious non-capsular bacilli. Very few encapsulated bacilli were found in the spleen and the heart blood. We grew non-capsular

bacilli from the heart blood which could not be induced to form capsules.

The strain proved wholly apathogenic for rabbits: A total of 9 rabbits was infected with 1/20 to 1 slant agar culture each; the site of infection showed temporary, insignificant edema that disappeared shortly; all animals survived.

Avirulent strain "$A_{22}R$": This strain was obtained from virulent A_{22} by Sterne's method (16) — streaked on agar containing 20% horse serum and grown for 5 days in an atmosphere containing 55% CO_2. We prepared a sub-culture from the dry edges of the slimy colonies and provided for the homogeneity of the resultant culture. The R variant obtained in this manner did not form capsules under any circumstances. The strain proved practically avirulent: Of 6 guinea pigs infected with 1/4 slant agar culture, 4 survived. The animals that died from the infection (on the 6th and 7th day, respectively) showed severe subcutaneous edema at the site of inoculation. The strain grown from the heart blood of the animals failed to encapsulate under normal conditions and in an atmosphere of 25% CO_2. The virulence for mice was insignificant: 3 mice were infected with 1/100, 3 with 1/1,000 and 3 with 1/10,000 slant agar culture; one animal of each group died. The dead animals revealed strong edema at the site of inoculation; we were unable to demonstrate encapsulated bacilli in the edematous fluid either by direct morphological examination or by culture.

2. The technique of immunisation with edematous fluid. The fluid was procured according to Okudo's method (22); it is mixed with 3.8% sodium citrate solution in the ratio 1:5 and collected. The citrated edematous fluid is mixed with a few drops of toluene, shaken mechanically for several hours, stored in the refrigerator and filtered through a thin layer of loose cotton just before use. We were always able to sterilise the fluid in this manner. In some cases we used the preparation "merthiolate" instead of toluene (dilution 1:10,000) for the killing of bacilli present in the edematous fluid.

In immunisational tests with edematous fluid we instilled the injection under the abdominal skin, or, in the case of mice, under the skin of the chest.

3. Infection of the animals. Here the site used for immunization was carefully marked and infection was carried out at a point removed as far as possible from the former. Rabbits received the injection into the lumbar region, in the proximity of the vertebral column; mice were inoculated under the dorsal skin.

When bacillary cultures were used for infection, the 24-hour slant agar culture was washed with 10 cc of physiological saline just prior to infection, the suspension was placed in a vessel containing glass beads, emulsified for 10 minutes in the shaker and then diluted to the different strengths. Rabbits received 1 cc, mice 0.5 cc subcutaneously.

The "animalized" bacilli were obtained from the edematous fluid of rabbits or guinea pigs. These bacilli were controlled as follows: 0.01 cc of the citrated edematous fluid is uniformly streaked on a surface of 1 cm^2, fixed and stained. The average number of germs present in the field is then determined under immersion. Since the size of the field is known, the number of bacteria may be approximately computed in this fashion. After this orienting examination the proper dilutions of the edematous fluid were prepared and immediately injected. At the same time agar plates were poured with the fluid and the precise number of inoculated bacteria was established.

4. Immune sera. The sera containing the capsular antibody were prepared on the basis of previous experience (5). The anthrax immune serum furnished by Mr. Szelyes ("Phylaxia" Serum Institute, Budapest) served as pure C-serum (anthrax serum containing somatic antibodies).

5. The haptens of the anthrax bacillus: The P substance was completely pure preparation obtained from a culture of Bac. mesentericus. The C substance was produced according to Tomcsik and Ssongott's method (27).

6. Execution of precipitation: 0.4 cc antigen of different concentration was mixed with 0.1 cc serum each. The result was read after 2 hours of standing in the water bath of 37°C and storage in the refrigerator overnight.

The antibody content of the serum of immunized animals was determined by the "ring test." The rabbit's blood was withdrawn from the auricular vein, tubes with an interior diameter of 4 mm were filled with serum up to about 0.5 cm and covered with a layer of antigen. The tail end of mice was cut off and the flowing blood collected in a capillary vessel (4 cm long) with an internal diameter of 0.9-1.1 mm. The capillary vessels were fused at both ends and placed in the centrifuge. The serum was finally covered by a layer of P substance in 1:1,000 dilution.

7. Complement fixation: Total spatial content 2.5 cc; 4 units of hemolysin were used. Since rabbit sera immunized against anthrax frequently show a high-grade auto-inhibition, titration of the complement was conducted in the presence of 0.1 cc serum. We used 1.5 units of complement in our main tests. Each serum was adjusted with the neutral solution of P substance (1:5,000-1:320,000) and with anthracic edematous fluid (1:10-1:1,600).

Experiments.

Rabbits immunized with edematous fluid.

In the course of our tests we wanted first of all to clarify the question whether immunity of rabbits immunized with edematous fluid by Bail's method is connected with the presence of the capsular antibody. The edematous fluid used in immunization was obtained from rabbits

infected with the virulent strain A_{33}. In the two test series listed in Table I we treated 16 rabbits with repeatedly sterilized or freshly centrifuged rabbit edematous fluid. On the 16th or 9th day after the last inoculation, blood was withdrawn from the animals and examined for presence of complement-fixing substances with the "ring test;" next we infected the animals with different amounts of animalized bacilli (strain A33). We noted in these tests that the pure P and C substances (in 1:500 dilution) as well as the edematous fluid (in 1:3 dilution) was not precipitated by the serum of the immunized rabbits. The complement fixation reaction conducted with antigens also leads to a negative result. Despite the absence of antibodies, a high-grade immunity of the animals was demonstrated unequivocally (see Table I).

It may be asked now whether the demonstration of antibodies in the serum might not fail because they remained tied to the tissue. Since we were able, in previous tests (7,8) to neutralise the immunity of mice immunised passively with the capsular antibody by means of injecting small quantities of purified capsular hapten, we also tried to use this method in connection with rabbits immunised with edematous fluid. For this purpose we treated the immunised rabbits with purified capsular substance in different ways prior to infection. In the two test series summarised in Table II, we also failed, as in the tests of Table I, to demonstrate precipitating and complement-fixing substances in the animals' serum. The animals' immunity therefore was not influenced by subcutaneous, intravenous or intraperitoneal treatment with P substance.

The rabbits received 2-2 cc edematous fluid sterilised with toluene in intervals of 4-5 days. Infection took place on the 7th day after the last inoculation of edematous bacilli.

TABLE I.

Test #	Treatment	Date of infection	# of bacilli	# of animals	# of dead animals	survival time (days)
I.	13 Jan 1 cc, 15 Jan 1 cc	12 Feb	438,000	2	0	
	20 Jan 1.5 cc, 31 Jan 4 cc	1938	219,000	2	2	3,3
	sterilised(*), 26 Jan 1 cc		43,800	2	0	
	fresh(**) edematous fluid					
	sucutaneously					
	Untreated (control)		43,800	2	2	2,2
			8,780	2	2	2,2
II.	7 Feb, 11 Feb and 17 Feb	26 Feb	3,180,000	2	0	
	3-3 cc sterilised(***)	1938	638,000	2	0	
	edematous fluid subcut.		318,000	2	1	4
			63,600	2	0	
			6,360	2	0	
	Untreated (control)		318,000	2	2	2,4
			63,600	2	2	4,4
			6,360	2	2	4,5

(*) Edematous fluid treated with toluene.
(**) 120-150 live germs per cc.
(***) One half of the animals received edematous fluid sterilized with 1:10,000 merthiolate, the other half fluid sterilized with toluene. The animals were infected with edematous bacilli.

Since the serum of animals immunized with edematous fluid revealed no antibodies in vitro, we tested the possibilities of protecting mice with the serum of immune rabbits. We examined two sera. One of them (designation "Oe I") represents the mixture of the sera, obtained by bleeding, of the three rabbits of test series I that survived the infection. The second serum (No. 146) came from the fourth rabbit of the same test series; this animal had been hyper-immunized with fresh edematous fluid prior to bleeding: It received a total of 19 cc freshly centrifuged edematous fluid intravenously, distributed on 5 injections within 67 days. It had been carefully centrifuged and contained an average of several hundred germs per cc.

In order to evaluate the protective effect of these two sera, the test was conducted with the freshly obtained serum of a normal animal, as well as with the serum of a rabbit treated with killed, encapsulated anthrax bacilli, the latter serum containing copious amounts of P antibody. The test of immunity of the last serum (P XV) was made one month after its production.

TABLE II.

Infection of rabbits immunized with edematous fluid, treated simultaneously with P substance.

Test #	Rabbit	Treatment with P substance	# of germs	# of animals	dead animals	Survival time (days)
I.	immunized	4 times 2-2 mg(*) i.v. and subcut.	8,500	4	0	
	"	0	8,500	4	0	
	control	0	8,500	2	2	2,3
	"	0	850	2	1	5
	"	0	85	1	1	3
	"	0	17	1	1	5
II	immunized	Twice 2 mg(**) i.p. and 3 times 2 mg i.v.	6,000	5	1	13
	"	0	6,000	4	0	
	control	0	6,000	4	4	3,3,4,3

(*) The animals received 2-2 mg (total 16 mg) P substance subcut. and i.v. four days prior to infection.
(**) On 11 and 12 Apr 2-2 mg i.p., on 13, 14, 15 Apr 2-2 mg i.v. Infection on 13 April.

Table III shows that 0.5 cc of the relatively fresh rabbit serum No. P XV, containing the capsular antibody, sufficed to immunize mice with 100% assurance against a 200-fold lethal dose. The protective effect of this serum was felt even in connection with 0.1 and even 0.05 cc.

TABLE III.

The protective effect of serum containing anti-capsular immune bodies (No. XV).

Infective dose (slant agar)	Quantity of instilled serum in cc 0.5	0.1	0.05	0 (control)
1:10,000,000		3/3	3/3	6/3
1:1,000,000	3/3	3/3	3/1	9/0
1:100,000	3/3	3/2	3/0	6/0
1:50,000	5/5			
1:10,000	3/3			
1:5,000	3/3			

Serum subcutaneously under the skin of the chest. The serum precipitated the purified P substance in a dilution of 1:4,000,000.

Infection with a 24-hour slant agar culture of strain A22 after 16 hours.

The denominator shows the number of animals surviving the infection.

Table IV shows experiments with mice, using sera of rabbits immunized with edematous fluid (Oe I) and serum No. 146 of the hyper-immunized animal. The mice received 1 cc of each of the sera. As a control, the fresh serum of a normal rabbit was used subcutaneously, also in the amount of 1 cc each.

TABLE IV.

The protective effect of sera free of P antibody, obtained from immune rabbits.

Infective dose(*) (slant agar)	1 cc serum from Oe I	Oe II(**)	No. 146	Normal rabbit	None (control)
1:10,000,000	4/2	6/2		6/1	15/3
1:1,000,000	14/5	6/1	9/3	6/0	15/1
1:100,000	6/0	4/0	3/0	6/0	3/0
1:10,000	3/0				

(*) 24-hour culture of anthrax strain A22.

(**) Repeated tests after storage for ½ year. 1 cc of the serum under the skin of the chest, infection under the dorsal skin after 15-16 hours.

The sera of rabbits immunized with edematous fluid therefore did not have a distinct protective effect, although they did not prove to be entirely ineffectual. Of the animals treated with fresh serum and infected with a (single) lethal dose, 1/3 survived. In treating with normal rabbit serum, however, we did not find the least protective effect.

The test results described so far show that it is possible to produce, with regularity, a massive immunity in rabbits by means of edematous fluid obtained by infecting rabbits with a virulent anthrax strain and containing capsular antibody in considerable amounts (see Table VI). Antibodies could not be demonstrated in the serum, however, either with the aid of precipitation or complement fixation; moreover, the immunity of animals immunized in this manner could not be neutralized by treatment with P substance. Mice could not always be immunized against an anthrax infection with the serum of immune rabbits, or the protective effect was so low that its specificity must tentatively be doubted.

Immunization of rabbits with the R variant of the anthrax bacillus.

In these tests we used two R strains that were completely apathogenic for rabbits; immunization was carried out successfully: Each of 6 rabbits was immunized with 3 doses of ½ slant agar culture of the live strain "R-1190". The animals showed a high-grade immunity against infection induced with animalized bacilli of strain A33 (see Table V).

TABLE V.

Rabbit immunization with strain 1190 R.

Treatment	Infection (# of germs instilled)	# of infected animals	Died
Vaccinated	2,600	2	0
"	13,000	2	0
0 (control)	130	1	0
"	1,300	1	1
"	13,000	1	1
Vaccinated	130,000	2	0

Immunization: The animal received ½-½ slant agar culture on 18 Mar, 2 Apr and 12 Apr. Infection on 25 apr with different quantities of edematous bacilli.

The animals listed in Table VII were treated partly with strains "R 1190" and A_{22}-R, partly with edematous fluid; the edematous fluid was obtained by infecting guinea pigs with strain A_{22}-R and sterilization in a merthiolate solution (1:10,000); later these animals were infected with virulent bacilli. Serologic reactions conducted with this edematous fluid yielded a striking proof for the validity of the assumption that the capsular antigen plays no role in the active immunity of rabbits.

For results of the serologic reactions, see Table VI. For purposes of comparison, the table also shows the results of serologic reactions obtained from edematous fluid produced by infection with a virulent strain. As revealed by the table, somatic antigen was demonstrated in both edematous fluids. The specific capsular substance, on the other hand, was found only in the edematous fluid caused by the effects of a virulent strain; the fluid produced by the R variant did not contain the specific substance.

TABLE VI.

The precipitation of various anthracic sera with edematous fluids from guinea pigs inoculated with virulent (A33) and avirulent (1190 R) strains of anthrax.

Origin (strain)	Serum	Dilution of the edematous fluid 1/4	1/8	1/16	1/32	1/64
1190 R	P	-	-	-	-	-
	C	+++	+++	++	++	+
A33	P	+	++	+++	+++	+++
	C	++	++	++	+	-

The results listed in Table VII show that immunization is invariably successful with both the R variant and the edematous fluid provoked by it. The edematous fluid, which could not be shown to contain the capsular substance even by serologic means, thus proved to be a very effective antigen and it seems that it is no less valuable in this sense than the fluid developing after infection with virulent bacilli.

TABLE VII.

Immunization of rabbits with anthrax strains A22R and 1190 R and with guinea pig edematous fluid free of capsular substance.

Treatment	Infective dose (slant agar)	# of animals	# of dead animals	survival time in days (average)
3 times A22R vaccine	1/50	7	0	
ditto	1/100	5	0	
3 times 1190R vaccine	1/50	3	0	
3 times 2 cc edematous fluid	1/100	5	0	
ditto	1/1,000	2	0	
0 control	1/10,000	3	3	3,3
ditto	1/5,000	5	4	5
ditto	1/2,000	4	4	3,5

Treatment: For 7-10 days ½-½ slant agar culture from the strains, 2-2 cc of the edematous fluid subcutaneously. Infection: With strain A33 on the 7th-11th day after treatment

Immunization of mice with edematous fluid.

According to previous observations (20,22) it might be assumed that active immunization of mice against anthrax either does not succeed at all or is possible to a limited extent only. In order to complement existing investigations in this field, we tried to immunize mice with sterilized edematous fluid. The rabbit or guinea pig fluid used here had been obtained by infection with strain A33; it was the same that we had used for the fairly successful immunization of rabbits. The results of the mouse test are listed in Table VIII.

TABLE VIII.

Immunization of mice with edematous fluid.

Test #	Treatment	Infective dose (slant agar)	# of animals	Died	Average survival (days)
I	4 times 0.2 cc guinea pig edemat. fluid	1:10,000,000	2	2	6
		1:5,000,000	2	2	7
		1:1,000,000	2	1	6
		1:500,000	3	1	10
		1:100,000	3	3	8
	0 (control)	1:10,000,000	3	2	7
		1:5,000,000	3	3	7
		1:1,000,000	3	3	4
II	5 times 0.03 cc rabbit edematous fluid	1:10,000,000	6	5	5
		1:1,000,000	6	6	5
		1:100,000	6	6	4
	0 (control)	1:100,000,000	3	1	4
		1:10,000,000	3	3	4
		1:1,000,000	3	3	4

The treatment of the mice was conducted with edematous fluid sterilized with toluene in intervals of 3-5 days. Infection took place on the 10th day after treatment, with a 24-hour culture of A22.

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A total of 30 mice was inoculated 4 or 5 times with 0.2 and 0.03 cc each of the guinea pig or rabbit edematous fluid. The amount of fluid dispensed to a mouse weighing 20 g therefore is 4 x 20 cc or 5 x 3 cc when applied to a rabbit of 2,000 g. This quantity (and even considerably smaller ones) invariably sufficed to immunize rabbits.

These results show distinctly that mice cannot be immunized with edematous fluid. In the tests we were able to keep only 3 of 30 mice alive; their death was to be expected on the basis of lesions observed in control animals.

Immunisation of mice with killed, encapsulated anthrax bacilli.

Judging from tests discussed so far, the capsular antibody seemed to play the decisive role in mouse immunity. We therefore investigated whether immunisation of mice succeeds with killed, encapsulated anthrax bacilli, assuming that the mouse organism itself would produce the capsular antibody. Numerous tests in this sense failed: The production of capsular antibody in mice could be elicited neither by subcutaneous nor by intraperitoneal treatment; thus the animals could not be immunized against anthrax infection. In these tests we treated a total of 86 mice in 5 test series partly subcutaneously, partly intraperitoneally, or both ways, in intervals of 2-3 days, 8 to 10 times with 1/5 to 1/20 slant agar culture of encapsulated, killed bacilli. The vaccine was produced from agar cultures of three different, strongly encapsulated strains and killed partly by heating to 100°C for 10 minutes, partly by treatment with 0.5% formalin. On the 7-10th day after the last inoculation, blood was withdrawn from the tail end in order to establish the presence of capsular antibody therein. We made the precipitation test ("ring test") with the blood and the purified and diluted (1:1,000) P substance, and infected the animals on the next day with the 10 to 20-fold lethal dose of the moderately virulent strain A15.

Among the 8 mice immunised in one test series, 7 revealed a weak precipitation. Five of these 8 animals survived infection with a 20-fold d.l.m. The vaccine killed with formalin was first dispensed subcutaneously, then intraperitoneally.

The remaining tests were not as successful: Among the remaining 76 mice the presence of antibody in the blood could be demonstrated positively in only 2 cases. An additional 8 animals showed a questionable result, i.e. the presence of capsular antibody could neither be demonstrated nor excluded. Of 76 mice, 16 survived the infection; 5 animals survived the 20-fold dose and 11 the 10-fold d.l.m. Of 25 control animals treated with the 20-fold d.l.m. and 8 treated with the 10-fold d.l.m., 2 survived in each group.

Due to the inconclusiveness of results, the question raised at the outset cannot be answered positively on the basis of investigations described here. As shown by the main part of the results, the mouse either cannot be led at all to form the capsular antibody by subcutaneous and intraperitoneal inoculation of killed, encapsulated anthrax bacilli, or will do so to a very limited extent; for this reason the attempt to immunise these animals against anthrax infection either fails completely or the result is uncertain.

Discussion of results.

We shall attempt to discuss the question of the mechanism of anthrax immunity on the basis of our test results which partly confirmed existing knowledge, partly added to it; in this connection we shall concentrate on the following problems: 1. What is the function of the individual

antigens of the bacillary body? 2. In which way do these affect the different animal species, primarily the mouse and the rabbit?

The tests by Tomcsik and Bodon (6) as well as Tomcsik and Ivanovics (7,8) previously discussed, established without a doubt that the immunity of the mouse is connected with the capsular antibody. As already mentioned, these authors noted that the capsular antibody confers a very low, practically non-existent immunity on the rabbit. On the other hand, rabbits may be successfully immunized with a live, avirulent, non-capsular vaccine (R variant), as described by Stamatin and Stamatin (22) as well as Sterne (16,24). There is absolutely no connection between the possibility of immunizing a species and the condition of the bacillary capsule, since rabbits may also be easily immunized by the subcutaneous injection of fluids in which the specific capsular substance cannot be demonstrated even by extremely sensitive serologic methods. In contrast to the absence of the capsular substance, the presence of the specific somatic substance may be positively demonstrated in this edematous fluid; the conclusion therefore offers itself that the immunity of the rabbit might depend on the specific substance of the bacillary soma. For the time being the possibility should not be ignored (and this has not been negated by investigations to date) that the specific substance of the bacillary soma might not represent the causative stimulus of immunity, but that "aggressins" have a function here, formed, according to Bail and his coworkers, by the bacilli in the living organism. In our opinion this possibility is the more plausible since we were unable to find antibodies — in the general sense (precipitin, complement fixing substances) — in the blood of rabbits immunized with edematous fluid and live bacilli. One of the additional missions of research must be the clarification of the question whether there really are "aggressins" in the sense of Bail, or whether the immunity evoked by live bacilli or edematous fluid depends only upon the bacillary somatic antigens going into solution in the living organism.

Rabbits immunized with edematous fluid or live bacilli showed the property that their immunity could not be transferred passively to mice, or that the rabbit serum confers a very limited protection on mice. On the other hand, as noted by Tomcsik and his colleagues, rabbits that had been consistently treated with intravenous injections of killed, encapsulated anthrax bacilli and the blood serum of which revealed considerable quantities of capsular antibody, were hardly more resistant to infection than normal animals; their serum, however, confers a high-grade protection on mice. Thus it seems that the presence of capsular antibody is significant only for mice, but not for rabbits.

Attempts to immunize mice by subcutaneous and intraperitoneal injection of killed, encapsulated anthrax bacilli were invariably unsuccessful. The cause of this failure seems to lie in the circumstance that the capsular antigen (which possesses relatively weak antigenic properties even for the rabbit) under these conditions either has no effect or very little. At any rate, this possibility is indicated by the circumstance that there were very few mice with traces of capsular

antibody in their blood. In these few cases the animals showed a somewhat stronger resistance to infection than untreated animals.

The question why attempts at immunization of mice with edematous fluid are so rarely successful, cannot be answered on the basis of the tests described. To date it is not clear whether the cause of this phenomenon is to be found in the lower reaction power or in the circumstance that the stimulating effect of the edematous fluid is inhibited by the peculiar protective mechanism of the mouse. The correctness of the last assumption is perhaps supported by the fact that it is easy to immunise these animals with the help of the capsular antibody.

It seems of interest to compare the results of tests described here with experience gained in pneumococcal immunity. The mechanism of the latter is sufficiently known since the extensive investigations of American researchers, and is suited to serve as the basis of knowledge in the field of immunity to encapsulated bacilli. As is well known, the somatic antigen and the capsular antigen of pneumococci have different functions with respect to immunity. Immunisational tests conducted by Avery and Goebel (29) with capsular polysaccharide (chemically tied to protein and thus turned into an antigen) proved most distinctly that the capsular antibody has a decisive function in the immunity of the mouse. Mice were successfully immunised against infection with the blood serum of rabbits immunised with the aid of this synthetic antigen; the blood serum contained only one pneumococcal antibody, the capsular antibody. As in the case of anthrax, the mouse can be immunised only with the capsular antibody. However, in the case of the pneumococcus the capsular antibody has an extremely important function also in connection with rabbit immunity, since this antibody alone also confers sufficient immunity on the rabbits. Thus a striking difference exists in this sense between immunity to pneumococci and immunity to anthrax.

However, there is also a similarity between the two immunities, in that the rabbit may be successfully immunised with the bacillary soma against both microorganisms. By intravenous treatment (30) with non-capsule- pneumococcus (R variant) or by intracutaneous inoculation with the encapsulated coccus, the rabbit organism is caused to develop only the antibody corresponding to the somatic antigen. Animals treated in this manner possess a very strong type-specific immunity. Mice cannot be immunised with the serum of these rabbits, as is true also in the case of anthrax (30).

It is therefore indispensable in research in the field of immunity to anthrax, to devote particular attention to each of the individual antigenic complexes of the bacterium. In the immunity against encapsulated bacilli the defensive mechanism of the mouse seems to be directed principally against the bacillary capsule, while the defensive apparatus of the rabbit would tend to become effective by way of the bacillary soma.

Summary.

1) The immunity of rabbits inoculated with sterilized edematous fluid fails to show any connection with the capsular antibody. The author was unable to demonstrate capsular antibody in the blood of immunized animals; attempts to neutralise the immunity of the animals with purified capsular antibody failed. Mice inoculated with 1 cc of blood serum of an immunized rabbit failed to show an important increase in resistance to anthrax infection over untreated controls.

2) Rabbits may be successfully immunized with the non-capsular R variant of the anthrax bacillus, as noted also by Stamatin and Stamatin, as well as by Sterne.

3) Rabbits may also be successfully immunized with the edematous fluid of guinea pigs killed by infection with the R variant; this edematous fluid fails to reveal the specific substance of the anthrax bacillary capsule even by use of very sensitive serologic tests.

4) Mice could not be immunised wither with the edematous fluid or with large quantities of killed, encapsulated anthrax bacilli.