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A Review of Selected Problems of Tularemia

in the Soviet Union

by

Dr. Robert Pollitzer

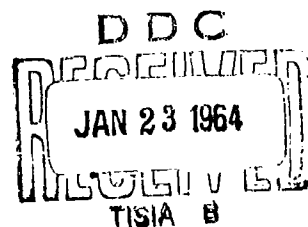
Part II/A

Vaccines and Vaccination

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The following Corrigenda to Tularemia, Part II/A were noted after the material had gone to press. Readers are asked to make the corrections in the present text. Ed.

<u>Page</u>	<u>Line</u>	<u>Should Read</u>
1	36	...(1960 <u>b</u>)...
2	15	...(1941 <u>c</u>) .
8	6	...(1941 <u>c</u>)...
11	25	...Olsuf'ev (1960 <u>b</u>)...
11	31	Shipitsina herself also found... (not "reported")
13	27	...consisted of malaise,... (not "debility")
24	4	...and slight malaise. (not "debility")
32	24	...of the <u>body</u> temperature,...
35	17	...had been suspended... (not "dissolved")
37	30	...(1958 <u>a</u>)...
37	42	...1957 (<u>see p. 21, supra</u>),...
38	31	...hitherto adopted... (not "used")
39	16	...(1958 <u>a</u>),...
39	25	Olsuf'ev (1960 <u>b</u>)...
41	46	...(1941 <u>a</u>) .
44	3	...(1941 <u>c</u>),...
44	14	Gaiskii (1944 <u>b</u>)...
44	45	...tularin tests in the...
45	23	...as the editors...
47	2	...1958 <u>b</u>)...
52	23	...(1960 <u>b</u>),...
53	22	...(1960 <u>b</u>),...
53	37	...Olsuf'ev (1960 <u>b</u>)...

<u>Page</u>	<u>Line</u>	<u>Should Read</u>
55	29	... (1958) ... (omit "a")
56	36	... (1958) ... (omit "b")
60	22	...Gefen and Gordon... (invert names)
61	11	(b) ... (not "a")
67	18	...described vaccine,... (omit "s")
68	7	...seems to have been used first <u>in 1955</u> ...
69	30	<u>Malaise</u> and Headache (not "Weakness")
70	9	...the authors...
71	5-10	"The local reaction after immunization was little marked in some persons, consisting of a slight swelling and redness at the site of the scarifications;...a general reaction (consisting of an enlargement of the cervical and axillary lymph nodes) was observed rarely--in 0.91%, and reactions in the form of fever or malaise were absent.
	Substitute:	
71	18-28	(3) <u>Tularemia and plague</u> . - The results of observations on simultaneous immunization against tularemia and plague recorded by Kalacheva (1958, 1959, 1960) may thus be summarized:....
	Substitute:	
76	23	...than the method of determining the survival rate of the animals... (insert after "vaccination")
77	28	...1959 a, c),...
87	37	<u>Total</u> <u>383</u> (not "283")
91	14	...combined... (not "simultaneous")
100	4	"The combined simultaneous vaccination...(invert)
104	8	Olsuf'ev (1960 <u>b</u>),...

A REVIEW OF SELECTED PROBLEMS OF TULAREMIA IN THE SOVIET UNION

II. Vaccines and Vaccination

A. Killed and chemically prepared vaccines

A discussion of the use which has been made in the Soviet Union of killed and chemically prepared vaccines is of historical rather than of practical interest, not only because the administration of these prophylactics has been given up entirely in favor of that of live vaccine but also because, as will be seen below, the investigations on their efficacy barely passed the laboratory stage.

According to the writers dealing with the history of tularemia vaccination in the Soviet Union, as for instance Sil'chenko (1955)¹, activities in this field had been started in 1931 by Khatcnover and his assistants Levchenko and Sinai. It would seem, however, that the first Soviet workers who demonstrated the possibility of an active immunization of laboratory animals against this infection were Suvorov and his associates (1928). It was these pioneers, as has been noted in the first part of the present review, who found agglutinins in the serum of guinea-pigs which had been immunized through the administration of a suspension of tularemia-infected materials inactivated by heating at 56°C.

An early attempt to produce an active immunity against tularemia seems to have been made by Zarkhi but, as quoted by Sinai (1935), he met with failure when challenging a guinea-pig which had been injected with material from a tularemia-affected spleen attenuated through long storage in glycerol.

In order to depict adequately in the present study the systematic attempts to use killed vaccines or chemically produced antigens for active immunization against tularemia, it seems preferable to deal seriatim with the various prophylactics used instead of reviewing chronologically the contributions made by the workers in this field.

1. The monograph by Maiskii "Immunology of tularemia. Theory and practice of vaccino-prophylaxis" (Moscow, 1953), in which according to Olsuf'ev (1960) the early history of tularemia vaccination in the Soviet Union was dealt with in an elaborate manner, was not available to the present reviewer.

Tularemia: I/2

Though, according to Sil'chenko (1955), Miller and his associates in the Rostov (Azovo-Chernomorskii) Institute of Microbiology and Epidemiology also worked with sisels (Citellus pygmaeus), as a rule white mice, guinea-pigs and rabbits were used for the early studies on tularemia vaccination in the Soviet Union. A point of great importance for the evaluation of the results then obtained is that, as stressed by Olsuf'ev, some of the early workers like Sinai and Khatenever and Levchenko (1935, 1938) used tularemia cultures which were not fully virulent to challenge their test animals.

1. Heat-killed vaccines

Experimental studies to evaluate the efficacy of heat-killed tularemia vaccines were made by Khatenever and Levchenko (1935), Burgasov, Vereninova (1936) and by El'bert and Gaiskii (1941).

Khatenever (1946), summarizing the results he had obtained with Levchenko, stated that they used a vaccine heated for 1 hour at 60°C five times in doses increased from 250 million to 1 billion organisms for the immunization of guinea-pigs and re-vaccinated the animals one month later with a one-billion dose of their vaccine. In spite of this energetic immunization all test animals died when challenged 1-2 months later with 200 DLM of a virulent tularemia culture.

As Khatenever added, better results were obtained by Burgasov¹ when experimenting with rabbits, animals far more resistant to subcutaneous tularemia infection than guinea-pigs or white mice (see Khatenever, 1943 a). The vaccine used on this occasion had been twice heated at 60°C for one hour and was administered 3 times in doses of 250 million, 500 million and 1 billion organisms. Out of the five animals thus immunized two withstood challenge.

Another group of rabbits immunized in the same manner were revaccinated 90 days later. When challenged 27 days afterwards through the subcutaneous administration of 20 million virulent tularemia bacilli, 7 out of the 10 test animals survived, while 11 out of the 15 controls died.

Vereninova (1936) used besides other vaccinal products (see below) one-day old egg-yolk tularemia cultures killed through

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1. The only two publications in point by Burgasov known to the present reviewer appeared in 1950 to 1951.

heating at 60°C for 1 hour for the immunization of rabbits. Since her intentions was to obtain the serum of the animals for diagnostic and therapeutic purposes, she administered this vaccine intravenously 4-5 times in doses increased from 500 million to up to 4-8 billion organisms. In contrast to the other vaccines used, that obtained by heating produced in the test animals sera with a low agglutinin titer (1:400). However, when used for neutralization tests in white mice, only one out of the four animals injected simultaneously with 0.2 ml of the immune rabbit serum and 10-10,000 DLM of a virulent tularemia culture succumbed.

The results obtained by El'bert and Gaiskii when trying to immunize white mice with 3 doses of tularemia vaccines prepared by heating at 60° or autoclaving at 120°C were altogether disappointing.

2. Agar-depot vaccines

As summarized by Khatenever (1943 b), the workers of the Rostov Institute of Microbiology and Epidemiology reported at the 1934 All-Soviet Conference of Microbiologists¹ that they had tried to enhance the efficacy of heat-killed tularemia vaccine by the incorporation of agar. For this purpose they used saline suspensions of B. tularensis with a concentration of 2 billion organisms per ml to which after heating at 60°C for 1/2 hour 0.5% sterile agar was added. This depot vaccine was twice administered intramuscularly to rabbits, each time in a dose of 2 billion organisms, and one month afterwards the animals were subcutaneously challenged with 4 billion doses of virulent tularemia bacilli. Whereas the controls succumbed to typical tularemia after an average of 4.7 days, only 5 out of the 10 immunized animals died after an average of 28 days. Only three of these rabbits yielded tularemia cultures and the gross changes found at autopsy in four of them were not marked. The survivors had shown no reaction to the vaccination and underwent no loss of weight. Since, however, some of them developed abscesses at the site of injection, Miller and his associates considered the use of glycerol vaccine (see below) preferable.

3. Chinosol vaccine

A vaccine prepared by Khatenever and Levchenko (1935) by the addition of 0.1% chinosol to suspensions of tularemia

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1. It is regrettable that neither this report nor a further report on this work by Grzhebina (1937) have been available for the purposes of the present review.

Tularemia-II/4

bacilli and apparently administered three times at intervals of 5 days, protected 5 out of 7 guinea-pigs against challenge with 10 to 100 million doses of a virulent tularemia culture. The sixth animal survived for one month, the seventh for 3 months.

No further details on the use of this vaccine could be found and it would appear that no further trials were made with it.

4. Glycerol vaccine

Referring in his 1943 summary to the use of glycerolized tularemia vaccines, Khatenever pointed out that in 1931 his associate Levchenko had started studies on the influence of glycerol on tularemia cultures with a view to using this chemical for the preservation of the antigenic properties of the organisms during the process of heating the bacterial suspensions in the course of vaccine manufacture. An addition of 3% glycerol before exposing the suspensions to a temperature of 60°C for one hour was found useful for this purpose.

In their initial investigation of a vaccine prepared according to this method with a concentration of 500 million organisms per ml, Khatenever and Levchenko (1935) immunized guinea-pigs 3 times at 5 day interval. Out of the 12 animals vaccinated in this manner one died 5 days after challenge, a second after 17 days, 5 were sacrificed after 15-23 days and five survived.

In a further series of tests the two workers immunized guinea-pigs 5 times at intervals of 4-5 days with glycerol tularemia vaccine and administered one month afterwards a booster dose. Challenge with 200 million virulent tularemia bacilli two months after revaccination gave most disappointing results, since all 12 test animals succumbed to tularemia, two like the controls within 10 days, the others after 11-18 days.

Therefore, it was decided to replace the monovalent with a polyvalent glycerol vaccine manufactured with four strains. This was administered 3 times at 5 day interval in doses of 500 million, 750 million and 1 billion organisms. When the guinea-pigs thus immunized were challenged one month later with 5 DLM, three died like the controls from tularemia within 10-11 days, two after 17-20 days. One animal succumbed after 6 months to an extraneous infection and four survived.

Tularemia-II/5

Khatenever (1946), added that further experiments with the glycerol tularemia vaccine, undertaken by him in cooperation with Levchenko and Kartasheva for the purpose of histological studies (see Kartasheva, 1935) gave a survival rate of 50% in guinea-pigs challenged one month after immunization with 5 DLM. Results in animals challenged 3-8 days after immunization with 10 million virulent tularemia bacilli also proved satisfactory. The vaccine did not protect guinea-pigs against infection with 200 DLM.

For tests on mice Sinai (1935) used a vaccine prepared by suspending tularemia bacilli grown for 2 days on a egg-yolk medium in normal saline containing 3% glycerol and heating the suspensions for 1/2 hour at 60°C. He recorded the following results:

- (a) Out of 17 mice which had been injected once with a 150 million dose of this vaccine and had been challenged 12 days later with 1.5 lethal doses of tularemia bacilli, 8 died like the controls within a week, the others 8-10 days after infection.
- (b) Out of 38 mice injected 4 times at four day interval with small doses of the vaccine 9 succumbed like the controls within a week after challenge, 24 within the second week and one on the 18th day. The remaining 4 animals, when sacrificed on the 25th day after challenge, were free from infection and agglutination tests with their sera proved positive.
- (c) Out of 70 mice immunized 5 times with large vaccine doses ranging from 100 to 200 million organisms, 13 died within 5 days after challenge, 30 within 60-10 days, 9 within 11-15 days and 18 survived for 17-36 days.

Continuing these investigations, Khatenever and Levchenko (1938) made two series of tests. In the first 21 mice were injected 3 times with 50, 100 and 200 million doses of a polyvalent glycerol vaccine and challenged two weeks later with 5 DLM of a virulent tularemia culture. Thirteen of the animals died within 10 days, 6 during the second ten day period and only two survived.

In the second series of tests 150 white mice were used in batches of 30 animals to assess the efficacy of four monovalent glycerol vaccines and a fifth prepared with two of the strains in question, the vaccine doses ranging from

Tularemia-TI/6

50 to 200 million. During the course of immunization 62 of the animals succumbed. Their deaths were ascribed by the authors to a toxic action of the vaccines. When 83 of the survivors were challenged one month after immunization with 2 DLM, 24 died within the first ten days, 31 within the second and 28 survived. When these 28 animals were again challenged one month later with 15 million of virulent tularemia bacilli, 26 succumbed within 10 days and only two survived.

As can be gathered from the 1943 summary of Khatenever, the workers of the Rostov Institute of Microbiology used a glycerol tularemia vaccine containing 500 million organisms per ml for the immunization of rabbits. One month after this had been administered three times at intervals of five days in doses of 200 million, 500 million and one billion organisms the animals were challenged with 4 billion doses of virulent tularemia bacilli which killed the controls after an average of 4.7 days. Out of the 7 immunized animals 4 survived after challenge and 3 succumbed to tularemia after an average of 17 days.

5. Formol-killed vaccine

Experiments with formol-killed tularemia vaccines seem to have been made first by Vereninova (1936). For this purpose she added 0.2% formol to suspensions of tularemia bacilli grown either on egg-yolk media or on cystin agar and let the mixtures stand at room temperature for two days. The two vaccines prepared in this manner and a third variant obtained through addition of 3% glycerol as well as of formol to suspensions from cystin agar cultures were administered to rabbits 4-5 times at intervals of 5-6 days, first in a dose of 500 million organisms, then, depending upon the result of agglutination tests with the sera of the animals, in gradually increased doses of up to 4-8 billion organisms.

As in the case of her heat-killed vaccine (see above), Vereninova used the sera of the rabbits immunized with the formol vaccines for protection tests in white mice. The results of these, as well as of those of the agglutination tests, are shown in the following table in which, for the convenience of record, the findings made with other types of tularemia vaccines by Vereninova have also been entered:

Tularemia-II/7

<u>Type of Vaccine</u>	<u>Maximal Agglutination Titer in the Sera of the Immunized Rabbits</u>	<u>Results of Protection Tests in White Mice</u>
Heat-killed	1:400	One mouse out of 4 died after 22 days.
Formol-killed from egg-yolk medium	1:1,280	10 out of 13 mice died after periods up to 24 days.
Formol-killed and glycerolized	1:2,560	One mouse out of 4 died after 27 days.
Formol-killed from cystin agar	1:4,000	One mouse out of 13 died rapidly.
Heat-killed vaccine made in 50% glucose solution	1:1,280	All 4 test mice died after 7-10 days.
Heat-killed vaccine made in 50% saccha- rose solution	1:2,560	2 out of 4 test mice died after 7-10 days.
Live avirulent	1:800	2 out of 4 mice died after periods of up to 22 days.

N. B. The controls used in these experiments all succumbed within a week.

As far as these tests go, of the three formol-killed vaccines that prepared with the aid of cystin agar proved efficacious.

Khatenever and Levchenko (1938) used four mono-valent formol-killed tularemia vaccines for the immunization of a total of 150 white mice, 48 of which succumbed in the course of this process. When 97 of the survivors were challenged one month after the immunization had been completed, 30 died within 10 days, 26 during the following ten

Tularemia-II/8

day period and 41 (42.3%) survived. Khatenever (1946) added that out of 10 guinea-pigs immunized with formol-killed vaccine only one withstood challenge with 200 DLM.

Quite disappointing were the results of immunization of guinea-pigs with formol-killed tularemia vaccines as recorded by El'bert and Gaiskii (1941) and by Manolov (1943). Vereninova and her associates (1943), while also unsuccessful with a formol-killed vaccine prepared in the usual manner, reported that 3 out of 8 guinea-pigs immunized 3 times with such a vaccine to which a small quantity of agar had been added, one month later withstood challenge with 10 DLM of a virulent tularemia culture. However, 4 out of 5 animals immunized in the same manner succumbed when challenged 2 months later with 5 DLM.

As summarized by Khatenever (1946), Burgasov found that

"rabbits, immunized 3 times in doses of 250 million, 500 million and 1 billion (with a formol-killed tularemia vaccine) when challenged 3 months afterwards showed a satisfactory result: 6 out of 8 animals survived. After 90 days a part of the rabbits was revaccinated with a dose of one billion and 27 days later infected with 20 billion. All 9 rabbits survived."

When Khatenever (1943 a) found that rabbits were rather resistant to the subcutaneous injection of tularemia bacilli, he decided to challenge them by the intravenous route. Using this method to challenge rabbits which had been immunized 13 1/2 months previously with a mixture of 1 part of formol-killed tularemia vaccine and 2 parts of olive oil, he noted that all four animals protected in this manner were resistant to the infection. However, the animals were unable to withstand an intravenous re-infection with massive doses of tularemia bacilli made 35 days after the first challenge.

6. Sugar vaccines

Vereninova (1936) postulated that a preparation of vaccines in sugar solutions as well as the addition of 3% glycerol prevented a "denaturation" of the protein substances of the bacterial cells during the heating process.

Tularemia-II/9

She, therefore, used suspension fluids containing 50% glucose or saccharose for the manufacture of tularemia vaccines. However, as shown in the table inserted above, the sera of rabbits immunized with these vaccines, though showing high agglutination titers, signally failed in mouse protection tests.

Further use of tularemia vaccines of this type (called AD, i. e. "adenatured" vaccines by the Soviet authors) was made by Vereninova and her associates (1943) in tests on guinea-pigs. The results obtained with these preparations, as compared with those of other methods of tularemia immunization, are shown in the following table:

<u>Kind of Vaccine^a</u>	<u>Number of Test Animals</u>	<u>R e s u l t s o f</u>					<u>Challenge Tests</u>
		<u>Agglutination Tests^b</u>			<u>Tularin Tests^b</u>		
		<u>Nega- tive</u>	<u>Posi- tive</u>	<u>Maximal Titer</u>	<u>Posi- tive</u>	<u>Nega- tive</u>	
1. AD vaccine	10	2	8	1:100	3	7	3 survivals
2. Formol-killed	15	8	7	1:80	2	13	No survivals
3. Formol-agar	8	2	6	1:60	1	7	3 survivals
4. Lysate	14	6	8	1:160	2	12	2 survivals
5. Live avirulent	10	-	10	1:160	6	4	8 survivals

Remarks: (a) Vaccine 1-3 and 5 were given 3 times at intervals of 7 days in doses of 500 million, 1 billion and 2 billion, the formol-agar vaccine in quantities of 0.25, 0.5 and 1 ml.
(b) Agglutination and tularin tests were made immediately before challenge.
(c) Challenge was made one month after immunization with 10 DLM.

Evaluating these results, one must agree with the authors that (1) none of the killed vaccines proved satisfactory and (2) the efficacy of vaccination with an atypical live tularemia strain (see the following section of this review) was most remarkable.

7. Lysates

Referring to the investigations made in the Rostov Institute, Khatenever (1943 b) made the following statement:

Tularemia-II/10

"The workers of the Rostov Institute also attempted a local immunization against tularemia.¹ For antigens they used a partly lysed culture from a solid egg-yolk medium in soda and in water antigens.

The soda antigen was thus prepared: a 24-hour old culture of Bact. tularensis on an egg-yolk medium was washed off with a 5% solution of sodium carbonate to which 0.5% phenol had been added. To effect lysis, the resulting suspension was kept for 24 hours in the incubator at 37°C and was then agitated in a shaking apparatus for 6 hours. The water antigen was prepared in an identical manner with distilled water.... The antigens were tested for sterility by culture and experiments on white mice. The ready-made water antigen was mixed with an equal amount of glycerol, the soda antigen with vaseline.

Immunization was effected by rubbing in the antigens 3 times in the course of two days into the shaven abdominal skin of rabbits.... 5-6 days afterwards the animals were challenged by rubbing in a mixture of one loop of a pure tularemia culture and a small portion of the triturated organs of a mouse which had succumbed to tularemia and in which the causative organisms abounded. Controls were infected at the same time."

While two preliminary tests with these antigens proved unsatisfactory, the two following gave better results: whereas 4 out of the 5 controls succumbed to tularemia within a period of 9-45 days after infection, none of the 10 immunized rabbits died. However, all fell ill 1-2 days after challenge.

In the opinion of the Rostov workers their method (particularly the use of the soda antigen) was useful in so far as it prevented death of the test animals and rendered the disease milder.

1. This mode of vaccination seems to have been used also by Khatenev who, in a 1941 paper not accessible to the present reviewer, described a prophylactic ointment against tularemia.

The kind of culture lysates with which Vereninova and her associates worked could not be established. As shown in the table inserted above, this method of immunization in their hands proved as ineffective as that with killed vaccines prepared in the usual manner.

8. Thermo-extracts

Khatenever (1946) claimed to have obtained good results when using thermo-extracts prepared by boiling suspensions made from tularemia cultures and subsequent filtration through asbestos wool for the immunization of rabbits. All 6 animals protected with the filtrate withstood challenge with a virulent tularemia culture one month later.

This author also added that satisfactory results were obtained by Burgasov when rabbits were immunized three times with thermo-extracts in doses corresponding to 5 billion, 10 billion and 20 billion tularemia bacilli. Eight out of the 10 animals thus protected withstood challenge.

According to Khat ver, Maevskii's chemical analysis of the thermo-extracts showed that the specific agent present in them was similar in composition to the polysaccharid-lipoid complex obtained by Boivin from gram-negative bacteria.

9. Complete antigen

As summarized by Olsuf'ev in the standard work on tularemia which he and Rudnev edited, Maiskii (1953) recorded good results when he used the complete antigen prepared by Shipitsina¹ according to Boivin's method from a virulent tularemia culture. Immunization of guinea-pigs repeated three times with this substance protected animals against challenge with 1,000 DLM. Shipitsina herself reported that the complete antigen immunized guinea-pigs against tularemia infection. It proved most effective when admixed to lanolin, so as to create a depot.

1. The earliest papers by Shipitsina quoted in the book edited by Olsuf'ev and Rudnev appeared in 1955.

Tularemia-II/12

According to Olsuf'ev's account, Tinker and his associates (1955), in an article not available to the present reviewer, stated that

"rabbits, guinea-pigs and in part white mice, intracutaneously immunized with the complete antigen prepared from a virulent tularemia culture, according to Boivin's method, manifested a resistance to infection with doses of F. (Francisella) tularensis known to be lethal."

It was found, however, that the animals lost the immunity thus conferred after three months.

As further quoted by Olsuf'ev, (a) Belkina and Petrosian (1958) found that the antigen prepared according to the method of Larson (1951) was weakly immunogenic for white mice, while (b) Emel'ianova found this antigen quite effective when administered to white rats.

The fact that immunization with tularemia antigens gave results superior to those obtained with killed vaccines, was, in Olsuf'ev's opinion, due to

"a more complete extraction of the antigen from the bacterial cells and its lesser denaturation, and also its higher concentration in the preparations used for the immunization of the animals."

Concluding his masterly disquisition, Olsuf'ev stated that

"The work on the 'chemical' vaccines cannot be considered as completed and the methods of their production have not yet been exhausted. Investigations in this direction ought to receive further attention. It must be remarked that all antigens obtained were clearly less effective than the live vaccine in the laboratory--not to speak of the incomparably greater expediency in the use of the latter because of the cutaneous method of administration and its application in single doses. It is very probable that the lowered immunizing activity of the killed vaccines is related in some way to a disturbance of their colloidal state. A formation of the antigens into fully valent colloidal combinations might enhance their immunogenic power."

10. Use for human vaccination

As can be gathered from the article by Khatenever and Levchenko (1935) and the summaries of Khatenever (1942, 1943 b, 1946) and of Sil'chenko (1955, 1960), the first attempt to use killed vaccines for the prevention of tularemia in man was made in 1931 at the Ush-Tobe Station of the Kazakhstan, where an outbreak of the disease had taken place in the preceding year. Referring to this experience, Khatenever (1946) stated that

"In 1931 Levchenko and I, with the help of Sinai, vaccinated 41 persons subcutaneously with a killed glycerol vaccine; the vaccine contained 500 million organisms per ml and was given in doses of 0.5, 1 and 1.5 ml every 5 days.

The persons who received three injections showed a positive agglutination reaction in 17 out of 24 instances with titers from 1:25 to 1:200; those vaccinated twice showed positive only in 2 out of 11 instances.

In 24 out of the 41 vaccinated, after the first injection one observed a local reaction consisting of hyperemia and edema in an area of 5 by 4 cm. An analogous reaction was observed in 9 persons after the second injection and in 2 after the third. The general reaction consisted in debility, headache and a rise of the body temperature to 37-37.5°C.

All vaccinated were under our observation for 3 weeks, which permitted us to establish the innocuousness of the method. We could not evaluate its epidemiological efficacy, since no tularemia was evident in the raion at the time."

However, as Khatenever continued, an opportunity for such an evaluation was offered in 1944 in the Tiumen Oblast. To use his own words again,

"In the settlements destined for vaccination a complete survey of the population for tularemia was made. About 60% of the inhabitants

Tularemia-II/14

who reacted negatively in agglutination and allergic tests, were inoculated twice with glycerol vaccine in doses of 100 and 500 million....

Both the local and the general reactions after vaccination were not intense. No revaccination was done.

Altogether 2,087 persons were surveyed, of whom 1,134 were found to have suffered from tularemia, while the other 953 persons gave negative agglutination and allergic reactions....

Of the negative reactors, 598 were vaccinated while 355 served as controls.

Tularemia outbreaks were observed in almost all places where vaccinations had been given. A survey made to assess the epidemiological efficacy of the vaccination led to the detection of 58 persons who had suffered from tularemia in the autumn of 1944. They all belonged to the control group, which consisted of people who had refused vaccination and of persons arriving in the settlements after the vaccination campaigns.

A careful observation failed to detect even a single case among the vaccinated."

Assessing this good result, Khatenever suggested that the infective doses to which the people living in the natural tularemia foci were exposed, were probably well below those to which the laboratory workers were exposed.

Results obtained in the immunization of man with formol-killed tularemia vaccines were rather disappointing. As Khatenever (1946) summarized, Grzhebina and Tsvetkova used this kind of vaccine in a tularemia focus for the immunization of 46 persons who all had been engaged in the hunting of water-rats or the handling of their skins and who resumed their occupation after they had received the first vaccine dose. Of these 22 fell ill with tularemia, while 24 remained well, even though 18 of the latter had been exposed to the same risks of infection as the persons contracting the disease.

A second trial of formol-killed vaccine was thus described by Khatenever (1946):

"Together with Tsvetkova and Gorbunova I administered (tularemia) vaccine prophylactically to 12 scientific and higher technical workers of the tularemia laboratory. A polyvalent vaccine made from three strains was used....

In 9 of the vaccinated a reaction and a rapidly disappearing sensation of burning at the site of injection were observed. A weak general reaction was observed in four persons. In the course of immunization the vaccinated showed a positive agglutination reaction with titers from 1:10 to 1:40.

The laboratory workers were not permitted to handle infectious materials before completion of the vaccination. Later, of the 9 who worked with such materials, 4 fell ill with tularemia: two suffered from a moderately severe generalized ('typhoid') form of the disease, two had slight attacks. The five others, who were exposed to the same risks of infection, remained well."

Khatenever felt entitled to maintain that "the formol-killed vaccine was effective in a number of cases." However, this opinion was not shared by later authors. Tsvetkova (1944), for instance, maintained that this as well as all other killed tularemia vaccines had been found little effective or had at least not proven their efficacy. It is possible, however, that she was not yet aware of the Tiumen Oblast results, which showed that these vaccines were not invariably ineffective.

B. Live vaccines

Early experiences

The studies of El'bert and Gaiskii which eventually led to the adoption of live vaccines for the prophylaxis of tularemia in the Soviet Union, date back to the period of 1932-1936 (Olsuf'ev, 1960 a). It would seem, however, that the results of these initial investigations have been first

Tularemia-II/16

referred to in a series of articles appearing at the end of 1941 in the Zhurnal mikrobiologii.¹ The findings then recorded by these two workers and which are relevant to the present disquisition, may thus be summarized:

1. Serological studies (Report I, Zh. mikrobiol.) led El'bert and Gaiskii to the conclusion that virulent tularemia bacilli possessed both H and O antigens. Hand in hand with a weakening of the virulence of the strains for laboratory animals the content in H antigen decreased. Strains which were avirulent for rabbits and guinea-pigs, but still weakly virulent for white mice, still contained traces of H antigen. In the strains which had become completely avirulent for laboratory animals, only O antigen was present.

2. It was found (Report III) that rabbits which had survived experimental tularemia infection were highly resistant to re-infection with massive doses of tularemia bacilli or tularemia "virus" (i. e. suspensions of the spleen of tularemia-affected white mice). El'bert and Gaiskii postulated that the quite slight reactions following re-infection, which were not accompanied by a rise of the agglutinin titer, were of an allergic nature.

Observations on laboratory workers also showed that an attack of tularemia led to a marked resistance to re-infection. For instance Gaiskii himself, contracting a conjunctival infection in the laboratory a few months after he had recovered from tularemia, developed merely a short-lasting local process. Likewise he and a laboratory servant (who had also had tularemia) were hardly affected when exposed a few years later to tularemia infection by inhalation.

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1. On the mechanism of infection and immunity in experimental tularemia: I. (El'bert and Gaiskii) Serological analysis of B. tularensis: II. (Gaiskii and El'bert) B. tularensis in the classified system of microorganisms: III. (El'bert and Gaiskii) Reactions of rabbits to the repeated administration of tularemia microbes and tularemia virus: IV. (Gaiskii and El'bert) Analysis of the allergen: V. (El'bert and Gaiskii) To the method of titrating the therapeutic anti-tularemia serum: VI. (Gaiskii and El'bert) Experimental tularemia infection of guinea-pigs and white mice and repeated infection: VII. (El'bert and Gaiskii) On the immunogenic efficacy of heated and formalized preparations of tularemia bacilli.

Pointing out that laboratory animals surviving experimental tularemia infection were apt to harbor the causative organisms for a prolonged time, particularly in their bone-marrow, El'bert and Gaiskii inclined to the belief that the immunity following tularemia infection was of a "nonsterile" nature, falling into the category of premunition.

3. In the probably most important sixth report of their series Gaiskii and El'bert recorded findings made with a tularemia strain which, having been kept in the museum, had spontaneously lost its virulence (see Matskevich, 1952, quoted by Olsuf'ev, 1960 b).

This strain, if administered to guinea-pigs by the subcutaneous, intracutaneous or oral route or by inhalation, produced only fever lasting 5-12 days without any other impairment of the health of the test animals. The reaction following one or two further administrations of the strain were still slighter and the alterations in the organs of sacrificed animals were found to be of a benign character with a tendency to regression. 80-90% of the guinea-pigs which had received one to three administrations of this strain subcutaneously or by inhalation proved to be resistant to challenge with 1,000 to 10,000 DLM of virulent tularemia bacilli and also to challenge by inhalation.

White mice which had received three subcutaneous injections of this strain in doses of 10, 100 and 1,000 organisms at intervals of 8-10 days, showed a survival rate of 75% and all survivors were found to resist a subsequent challenge with 10,000 DLM. Thus, as Gaiskii and El'bert emphasized, the possibility of producing in white mice a solid active immunity against tularemia had been demonstrated for the first time.

As added by Matskevich, this slightly virulent but highly immunogenic "Moskva" strain, when subcutaneously administered in 50 million doses to 10 human volunteers, caused in general no marked reactions. When tested with tularin after 2-3 weeks, the vaccinated persons showed positive reactions and it was also found that their serum agglutinated tularemia bacilli in dilutions ranging from 1:20 to 1:160. However, as has been mentioned already, for reasons beyond their control El'bert and Gaiskii were unable to continue work with this strain which became eventually lost. The resumption of their studies on tularemia vaccination with live strains will be discussed after attention has been paid to work in this field by other early observers.

Tularemia-11/18

Mention has first to be made in this respect of Vereninova (1936) and Khatenever and Levchenko, the results of whose unpublished 1936 investigations have been summarized by Khatenever (1943 b).

Besides killed vaccines Vereninova (1936) used a live avirulent tularemia strain and also a live virulent culture for the immunization of rabbits. While most of her test animals well tolerated administration of the killed vaccines, not only the two rabbits receiving intravenous injections of the virulent culture (apparently in a dose of 500 million organisms) but also 4 out of the 6 animals injected by the same route with the avirulent strain rapidly succumbed. In this connection Vereninova recorded that

"We noted death of 2 (of these) rabbits immediately after the intravenous administration of the antigen, the others we found dead on the next morning. Though three animal passages were made, we could not isolate cultures from them. At autopsy one observed hemorrhages on the organs, inflated and not collapsing lungs, a normal state of the lymph nodes and hyperemia of the normally sized spleen. The hyperemia and hemorrhages in the organs, the spasm of the bronchial musculature and the signs of asphyxia were evidently the result of anaphylaxis. Later we resorted to desensitization, introducing a small dose under the skin and completing the immunization with the avirulent culture (by the intravenous route)."

As will be gathered from the table inserted on page 7 of this review, the agglutination titers in the sera of the rabbits vaccinated in this manner as well as the results of mouse protection tests made with these sera were inferior to those obtained with some of the killed vaccines. It is interesting that notwithstanding these disappointing results Vereninova, in the conclusions to her article, urged a continuation of the work with live tularemia cultures.

Referring to the experiments he had made in 1936 with Levchenko, Khatenever (1943 b) stated that in the course of this work they immunized guinea-pigs with killed vaccines and also with a live weakly virulent tularemia culture, used in doses gradually increased from 25 to 250 million organisms

Tularemia-II/19

for five injections made at intervals of 4-5 days. One month after the completion of this immunization a booster dose was administered. Challenge tests with 200 million doses of virulent tularemia bacilli were then made after 1-2 months. While with one exception the animals immunized with killed vaccines succumbed, apparently 9 out of the 14 guinea-pigs immunized with the live vaccine withstood challenge. Rather surprisingly, however, Khatenever did not comment favorably upon this result.

To judge from a brief remark by Sil'chenko (1955), Grzhebina (1937) obtained no satisfactory results when using live as well as killed tularemia vaccines for the immunization of various rodent species.

In their 1938 article already referred to earlier in this review (see page 5) Khatenever and Levchenko also reported on results obtained when immunizing white mice with four monovalent and one bivalent live tularemia vaccines. As in the case of their killed vaccines, they observed a high mortality (79 out of 150 animals) in the course of immunization. When the survivors were challenged one month after the third vaccine administration with 2 DLM, the results thus compared with those in the animals immunized with killed vaccines:

<u>Kind of Vaccine</u>	<u>Number Challenged</u>	<u>Died</u>		<u>Survived</u>	
		<u>10 Days</u>	<u>11-20 Days</u>	<u>Number</u>	<u>Percent</u>
Live vaccine	64	25	16	23	35.9
Glycerol vaccine	83	24	31	28	33.7
Formol vaccine	97	30	26	41	42.3

N. B. All survivors succumbed when infected one month after the first challenge with large doses of virulent tularemia bacilli.

In view of these results, the two workers were not favorably impressed by the live tularemia vaccines.

Experimenting with rabbits, Khatenever (1943 a) found that (a) 2 rabbits which had been immunized with live tularemia vaccines 14 months previously withstood intravenous challenge (dose ?) and (b) it was possible to enhance the natural resistance of these animals through subcutaneous

Tularemia-II/20

administration of sublethal doses of a weakly virulent tularemia strain. However, almost all the test animals succumbed when intravenously challenged after 35 days with massive doses (100 million or 1 billion) of the same strain.

As already recorded (see the table inserted on page 9 of this review), Vereninova and her associates (1943) obtained satisfactory results when immunizing a group of 10 guinea-pigs with a live tularemia vaccine, 8 of the animals surviving when challenged one month later with 10 DLM. As these workers stated, the growth of their vaccinal strain on egg-yolk media

"differed somewhat from a typical culture: it was considerably slighter and more often led to the formation of isolated colonies. The morphology of the bacilli was also somewhat peculiar: side by side with typical coccobacilli one found fairly large disk-like organisms. Guinea-pigs tolerated infection with 500 million, 1 billion and 5 billion of this strain without falling ill."

Though Vereninova and her co-workers stressed the superior efficacy of immunization with live tularemia vaccines, it seems that no further use was made of their strain, even though it had served well for this purpose.

Further fundamental studies

A new epoch in the history of tularemia immunization in the Soviet Union began in 1941 when Gaiskii succeeded in the artificial production of strains suitable for administration in the live state. As summarized by Emel'ianova (1960),¹ he kept for this purpose

"the cultures on a coagulated egg medium or in broth (with anti-serum) and subcultivated them at the limits of the almost complete dying off of the microbial populations, assuming that the decrease of the virulence stands in connection with the aging of the culture(s)."

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1. Regrettably the two publications of Gaiskii (1943, 1944 a) describing the production of these attenuated strains could not be consulted in the original.

As added by Olsuf'ev (1960 b), Gaiskii obtained through serial subcultivations made in this manner

"two attenuated strains--15 ("bul'onnyi, 17th generation) and Ondatra IV ("dry," 6th generation). The strain Ondatra IV was more attenuated than the strain 15. For instance, when inoculated subcutaneously with the first mentioned strain, white mice withstood doses of 500,000 and 5 million microbial cells and even a dose of 50 million did not prove certainly lethal (DCLM), whereas only 15-20% of the white mice given 5 microbial cells of the strain 15 survived and higher doses killed them all (Gaiskii, 1943). Guinea-pigs tolerated inoculation of the strain Ondatra IV in a doses of 500 million microbial cells but only 2.5 million doses of the strain 15 (higher doses were apparently not tested). The author called the first mentioned culture a strain with residual virulence, the second a weakly virulent strain. Pathological studies by Donskov (1944) on guinea-pigs vaccinated with Gaiskii's strain 15 showed a benign character of the inflammatory changes observable in the regional lymph nodes and partly also in the internal organs of the animals."

According to Olsuf'ev account, Gaiskii afterwards produced some further strains with a similar degree of residual virulence for laboratory mice. However, among all these cultures only the strain 15 proved fairly stable, the strain Ondatra IV for instance losing its valuable properties within a few years. Still, as will be discussed below, even the strain 15 had to be replaced eventually by a reconstituted variant and a new strain 155 (Emel'ianova, 1957 a, b).

Summarizing in an article published in the Zh. mikrobiol. in 1944 the results obtained up to then with his attenuated strains, Gaiskii stated the following:

"1. The attenuated strains at our disposal are highly immunogenic, protecting, if once injected subcutaneously in small doses of 1-5 organisms, guinea-pigs and white mice against 1,000, 10,000 and 100,000 lethal doses of a virulent tularemia culture.

Tularemia-II/22

2. Single administrations of the attenuated strains produce in rabbits and guinea-pigs a clearly marked sensitivity to the tularemic allergen, what furnishes objective proof for the development of a high resistance to a virulent re-infection.
3. The attenuated strains with a residual virulence, as well as those which are weakly virulent for white mice and completely avirulent for guinea-pigs, are stable in their biological properties for the latter but not for white mice, in which a repeated passage produces an increased virulence.
4. The attenuated strains with a residual virulence for mice are apt to retain their immunogenic properties for 2 years (limit of observations).
5. Prolonged observations on vaccinated laboratory animals give the right to assert that there is no reason to fear a reversion of the attenuated tularemia bacilli into the virulent state during the process of immunization.
6. Observations on about 4,000 vaccinated people living under most variegated conditions led in no instance to the detection of ailments which were connected with the inoculations and gave no reason to speak of the possibility of any reversion of the strains.
7. It is possible to stabilize the immunobiological properties of the tularemia strains for a prolonged time through drying under a high vacuum.
8. The immunity after a tularemia attack as well as that produced artificially through the administration of attenuated strains are of long duration. A period of one year does not mark the limit of the length of immunity resulting from the administration of live vaccine.

9. Large-scale vaccination campaigns among the population of tularemia foci (6,652 persons) give the right to assert the innocuousness, immunogenicity and full suitability of the method as an obligatory prophylactic measure.

10. Experiences in animals as well as observations in people inoculated with the live vaccine testify to the development of an intense immunity against massive doses of virulent tularemia cultures."

The most spectacular proof for the last conclusion, referred to in the text of Gaiskii's article and also by Kosmachevskii (1944) and subsequent writers like Sil'chenko (1955) was furnished by a test on human volunteers made in 1942 at the Irkutsk Anti-Plague Institute. There, as described by Kosmachevskii, in June 1942 50 persons had been subcutaneously injected with live tularemia vaccine, 26 of them receiving doses of 5,000 organisms of the strain 15 and 24 various amounts of the vaccine prepared with the strain Ondatra IV--17 receiving doses of 500,000 organisms, 6 such of 50,000 organisms and one 5,000 organisms. Out of this group six persons were challenged at the end of 1942 with a virulent tularemia culture. Referring to this heroic test, Gaiskii (1944 b) stated that

"As controls 3 workers of the tularemia department were taken: one of them had had a severe tularemia attack 12 years before and two had asymptomatic tularemia in 1941 and 1942. Thus altogether 9 persons participated in the test, all giving a clearly positive tularin reaction.... The minimal lethal dose of the (challenge) strain used was 5 organisms. Out of the 9 persons one received 10 such lethal doses, five 100 doses and three 1,000 doses.

Out of the 9 persons eight showed a general reaction; a local reaction was also observed in 8 instances. As a rule the general reaction became manifest on the second day (after challenge).... The local reaction became manifest 1-2 days after infection and consisted of a painful swelling with reddening of the skin at the site of the injection and a slight enlargement of the regional lymph nodes (in 2 instances).

Tularemia-II/24

The general reaction consisted of an increase of the body temperature lasting from some hours to 2-3 days (in 2 instances), headache and slight debility. The whole experiment took 3 days."

As stated by Sil'chenko (1955), the splendid outcome of this test, which fully confirmed the findings made in experimental animals, immediately led to the actual use of Gaiskii's vaccine, 1,300 persons being inoculated under his supervision in the Kirov Oblast still in 1942, 2,214 persons in the Voronezh Oblast and 2,000 in Kazakhstan in 1943. The only drawback of Gaiskii's vaccine was that, if kept at room temperature, it remained potent for one week only. As alluded to above, Gaiskii tried to prepare his vaccine in dry form, but though this procedure considerably prolonged the keeping qualities of the product, if stored at a low temperature (0.2°C), the studies made in this direction were not completed (Sil'chenko, 1955).

In an important study on the "Rapidity of appearance and length of persistence of the immunity after vaccination with live tularemia vaccine," published by Gaiskii and his associates in 1947, the following findings were recorded:

I. Onset of Immunity

- (a) Results in white mice once subcutaneously injected with 100 organisms of a tularemia strain with residual virulence and challenged with 10,000 DLM of a virulent culture:

<u>Time of Challenge</u> <u>(Days after Vaccination)</u>	<u>Number of</u> <u>Test Animals</u>	<u>Survived</u>	<u>Died</u>
5	16	7 (43.7%)	9 (surviving the controls for 1-6 days)
10	19	All	-
15	19	All	-

Tularemia-II/25

- (b) Results of allergic tests in guinea-pigs once subcutaneously injected with 1 million doses of the above mentioned vaccinal strain:

<u>Time of Test</u> <u>(Days after Vaccination)</u>	<u>Number of</u> <u>Test Animals</u>	<u>Results of Tularin Test</u>
5	4	All negative
10	4	2 weakly +, 1?, 1 -ve
15	4	All ++
21	11	7 ++, 4 +

- (c) Results in guinea-pigs vaccinated with 1 million doses of the same strain and challenged with 100,000 DLM of a virulent tularemia culture:

<u>Time of Challenge</u> <u>(Days after Vaccination)</u>	<u>Number of</u> <u>Test Animals</u>	<u>Survived</u>	<u>Died</u>
5	5	2	3 (surviving the controls for 3-4 days)
9	7	4	3 (surviving the controls for 6-13 days)
22	8	7	1
30	-	-	Almost 100% survival in a large-group.

(It thus appeared that the onset of a general immunity antedated the appearance of a positive allergic reaction.)

- (d) Results of agglutination tests in 10 guinea-pigs vaccinated with 1 million doses of the same vaccinal strain:

Tularemia-II/26

<u>Time of Test</u> <u>(Days after Vaccination)</u>	<u>Number of</u> <u>Test Animals</u>	<u>Results</u>
10	10	Positive (one plus) in a dilution of 1:10 in 5 animals.
26	-"-	7 times ++ or +++; ? once, -ve twice (maximal titer 1:10).

(The authors concluded from these findings that evidently "the agglutinins play no substantial role in the immunity against tularemia.")

(e) Results of tularin tests in vaccinated persons:

<u>Vaccine Dose</u>	<u>Time of Tests</u> <u>(Days after Vaccination)</u>	<u>Number</u> <u>Tested</u>	<u>Positive</u> <u>Results in</u>
5,000-500,000	8-12	59	22 (37.2%)
Dto.	21-40	43	30 (75%)
20 million	5	10	8
Dto.	20	8	All positive (6 ++)

(In the vaccination campaigns conducted with a dosage of 20 million organisms allergic tests made 30 days after inoculation gave almost invariably a positive result.)

(f) Results of agglutination tests in vaccinated persons:

<u>Vaccine Dose</u>	<u>Time of Tests</u> <u>(Days after Vaccination)</u>	<u>Number</u> <u>Tested</u>	<u>Results</u>
5,000-500,000	10	14	All negative (1:25)
Dto.	21-40	41	9 positive at titers from 1:25 to 1:100.
20 million	10	?	All negative (1:10)
Dto.	20	8	Positive at 1:100 once, at 1:25 3 times, at 1:10 four times.

(g) Results of opsono-phagocytic reactions:

It was found that

"The phagocytic reaction in the vaccinated becomes manifest very early, from the 3rd day. In the course of the first two weeks after vaccination with doses from 5 to 25 million organisms one may characterize the opsono-phagocytic reaction as weakly positive: in the 14 persons tested the opsono-phagocytic index varied within a range from 10 to 20. Within 30 days the index in the 13 vaccinated persons tested in this respect was higher than 50."

The general conclusion of the authors was that in man as well as in the laboratory animals an immunity response to tularemia vaccination began to become manifest within the first days following the administration of the live vaccine.

II. Duration of the immunity

- (a) Observations on white mice and guinea-pigs subcutaneously injected with 5,000 organisms of the vaccinal strain and challenged by the same route with 1,000 DIM of a virulent tularemia culture:

	<u>Time of Challenge</u> (Months after Vaccination)	<u>Number of</u> <u>Test Animals</u>	<u>Number of Survivors</u>
<u>White mice:</u>	1	8	8
	3	10	7
	4	24	3
<u>Guinea-pigs:</u>	5	8	8
	10	8	7
	15	5	3

- (b) Observations in man:

Considering their own findings as well as unpublished data of Altareva, the authors stated that

"in 20 persons who had been subcutaneously vaccinated once 2 1/2-3 1/2 years previously with live tularemia vaccine, the allergic reaction

Tularemia-II/28

"was well marked in 19, giving in one instance only a doubtful result. It is necessary to state that one month after vaccination in all 20 the reaction was clearly and well marked. The opson-phagocytic index was high in 12 out of these 20 persons, twice moderately high, 4 times low, two times negative. A positive result of both reactions was noted in 85%."

While as noted above, the endeavors of Gaiskii to produce a live tularemia vaccine in stable dry form had not led to a practical result, success in this respect was obtained by Faibich and Tamarina. As these two workers summarized in the first of their two initial publications dealing with this work, which was done under the auspices of the Institute of Epidemiology and Hygiene of the Red Army¹ (1946 a), investigations made with the two vaccinal strains of Gaiskii had shown

"that the drying of the cultures of the vaccinal strains, suspended in media which contain 10-15% saccharose, 0.1% agar and 1.5% gelatin, yields a dry preparation with a high content of live organisms capable of prolonged survival. Thus the biological titer of the suspensions of the organisms of the live vaccine in this medium equalled 10^{-8} or 10^{-9} and this titer was maintained after the drying. If this dry saccharose-agar-gelatin vaccine is kept in ampules under a vacuum at a temperature of $2-4^{\circ}\text{C}$ for a year, its biological titer undergoes no change. Storage of the vaccine at a higher temperature led to a decrease in the amount of live organisms in relation to the temperature. After a storage at 18° for 250 days or at 26° for 75-90 days the biological titer was 10^{-4} or 10^{-5} . The vaccine kept under these conditions, if given in 12 1/2 million doses, produced in guinea-pigs and white mice an immunity of high degree, ensuring a survival of 95-100% of the animals after challenge with 1,000 DLM of a virulent culture."

1. Nauchno-issledovatel'skii institut epidemiologii i gigeny or, in abbreviation, NIEG.

Tularemia-II-29

The second article by Faibich and Tamarina (1946 b), recording the results obtained with their dry vaccines, contained the following particularly noteworthy statements:

1. Experimental findings showed that "the strain Ondatra IV (was) almost avirulent for guinea-pigs and weakly virulent for white mice, the strain 15, on the contrary, more virulent for guinea-pigs and less so for white mice."

2. Single subcutaneous administrations of the dry vaccine prepared from the strain Ondatra IV in a dose of 125,000 organisms or in higher doses, or of the vaccine from strain 15 in doses of 6,000 organisms or more invariably protected guinea-pigs against subcutaneous challenge with 1,000 certainly lethal doses of a virulent tularemia culture. The vaccines also protected guinea-pigs against subsequent respiratory infection.

3. The two dry vaccines proved highly immunogenic in the case of white mice: single subcutaneous injections of the vaccine prepared from strain 15 in a dose of 1,000 organisms or of the Ondatra IV vaccine in a dose of 15,000 organisms invariably proved potent against subcutaneous challenge with 1,000 lethal doses and protected 65% of the mice against respiratory infection with a virulent tularemia culture.

4. The dry tularemia vaccines, if stored in sealed ampules for 270 days at 18°C or for 75 days at 26°, or at 2-4° for 1 1/2 years and used in single subcutaneous doses from 10 to 25 million organisms, still protected 95-100% of the test animals (guinea-pigs or white mice) against challenge with 1,000-2,000 lethal doses.

5. The dry vaccines (as well as the suspensions made directly from the vaccinal strains) were efficacious only if administered in doses causing a local reaction.

It was established in this connection that the minimal doses necessary to protect 90% of the animals were as follows:

<u>Species</u>	<u>Strain 15</u>	<u>Strain Ondatra IV</u>
White mice	10 organisms	1,000 organisms
Guinea-pigs	1,000 "-	10,000 "-

Tularemia-II/30

If given in lesser doses, the cultures of the vaccinal strains conferred an immunity to not more than 50% of the test animals. Gaiskii's claim that 1 to 5 organisms of his strains invariably protected the animals against challenge, was thus not confirmed.

6. The immunity conferred to guinea-pigs through administration of 12.5 million doses of the dry vaccine made from strain 15 began to become manifest after 3 days and became maximal 15 days after vaccination. Corresponding tests with the dry vaccine prepared with the Ondatra IV strain in doses of 25 million indicated an onset of the immunity after 8 days and its acme after 15 days.

There was no evidence for the presence of a negative phase.

7. Observations made first on 52 volunteers and then during vaccination campaigns on over 30,000 persons showed that, as long as dosages of 25 million organisms were not exceeded, the reactions following the administration of dry tularemia vaccines were mild in healthy individuals and not unduly severed in those who had suffered from the disease.

8. Since, as shown by the results of allergic and agglutination tests, the dry vaccine prepared from the strain 15 proved more effective than that for the manufacture of which the strain Ondatra IV had been used, Faibich and Tamarina recommended the administration of the former in doses of 10-25 million organisms. In their opinion it was under these conditions not necessary to exclude individuals with a history of tularemia.

9. Since the use of a polyvalent tularemia vaccine seemed desirable, a search for suitable strains was instituted. Two such strains (No. 10 and No. 33) were found and, as stated by Sil'chenko (1960), were thereafter used together with the strain 15 for the manufacture of the dry NIIEG tularemia vaccine.

A new phase in the history of tularemia vaccination in the Soviet Union was initiated in 1945 through the publication of an article by El'bert in which, as a result of investigations started in 1937, he drew attention to the possibility of using the method of cutaneous inoculation in place of the hitherto practiced subcutaneous administration of live tularemia vaccine. Experimenting on guinea-pigs, El'bert established that the

rubbing in of 0.1-0.2 ml of tularemia virus-vaccine containing 50-100 million organisms into skin scarifications rendered the animals resistant to challenge with a virulent tularemia culture by the cutaneous, subcutaneous, oral or respiratory route. He also obtained good results when for this purpose, instead of saline suspensions of the vaccinal organisms, he used suspensions in 50% neutral glycerol, 40% saccharose or hyposulfite solution so as to keep the vaccines potent for the period of one month. Likewise he found it possible to combine the cutaneous administration of smallpox and tularemia vaccines.

In a further publication, El'bert and his associates (1946) recommended for cutaneous tularemia inoculations the use of a vaccine prepared with the aid of the fluid egg-yolk medium of Drozhevskina (1945), in which the tularemia bacilli were apt to survive for periods of up to one year.

At the same time El'bert and his co-workers recorded further observations on the efficacy of the cutaneous method of tularemia vaccination in experimental animals, stating inter alia that

"Guinea-pigs (cutaneously) vaccinated 12 or more days before challenge, acquire an intense immunity to a virulent infection, i. e. they survive and usually show no increased temperature when injected with 10,000 lethal doses. This group of animals shows a positive allergen reaction. A second group--animals which were cutaneously vaccinated 3-11 days before infection and give a negative tularin reaction--also survives after injection of 10,000 lethal doses of a virulent tularemia culture but shows clinical signs of infection in the form of an increased body temperature. It may be asserted a change in the state of immunity (Umstimmung) begins to develop in the animals already soon after the cutaneous administration of live attenuated tularemia culture and that after 3-4 days the animals have acquired a partial immunity which after 12 days passes into a phase of complete readiness to encounter and overcome the infection."

Tularemia-II/32

In their 1946 article El'bert and his associates also reported satisfactory results of a campaign conducted with the aid of their method of immunization in 24 tularemia-affected settlements.¹

It was found on this occasion that cutaneous administration of the egg-yolk tularemia vaccine practically always led to a local reaction, which usually appeared on the 4th-6th day after inoculation. This consisted

"of reddening and slight swelling of the skin strictly along the line of the scarifications made with a lancet as used for smallpox inoculation, into which the egg-yolk vaccine had been rubbed in. After 2-4 days, following the formation of linear pink papules, there appeared small vesicles with a transparent content which then dried, becoming covered with crusts. Around the scarifications one could note a zone of congestion, as characteristic for smallpox vaccination. After 3-4 weeks the crusts fell off and thin scars appeared in their place."

In about 20% of the vaccinated persons one could note an enlargement of the lymph nodes draining the site of inoculation, which caused some discomfort and tenderness, but no pains. Apart from a slight rise of the temperature, observed according to El'bert et al. (1947 b) in 10% of the vaccinated, signs of a general reaction were as a rule absent and the working capacity of the recently vaccinated was never impaired.

Tularin tests made in 100 vaccinated persons 5 to 48 days after the inoculation gave positive results from the 7th day onwards. Agglutination tests with the sera of the vaccinated gave from the 18th day after inoculation onwards the positive results shown in the following table in which, for the sake of comparison, also the corresponding findings made in persons attacked 12 to 90 days previously by tularemia are inserted:

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1. A description of preliminary observations in two small groups of cutaneously vaccinated volunteers has been given in the first of a series of articles on this method of immunization published in the 6th volume of the Transactions (*Trudy*) of the Rostov Anti-Plague Institute (El'bert et al., 1947 a).

Tularemia-II/33

<u>Group</u>	<u>Number of Positive Reactions</u>	<u>1:20</u>	<u>1:40</u>	<u>Maximal Titers</u>				
				<u>1:80</u>	<u>1:160</u>	<u>1:320</u>	<u>1:640</u>	<u>1:1280</u>
Vaccinated	157	3	8	48	49	26	17	6
Recently tula- remia-attacked	77	-	-	2	9	13	25	28

As stated by El'bert and co-workers (1946), during the period of 50 days following the above mentioned large-scale vaccination campaign tularemia attacks were observed in 0.36% of the inoculated group as against an incidence of the disease in 4.3% of the almost equally large not vaccinated part of the population. Most noteworthy was also that (a) whereas tularemia attacks continued among the not vaccinated almost throughout the observation period, an incidence of the disease among the inoculated persons was noted only during the first twelve days following the administration of the vaccine; and (b) the disease ran a slighter course in the vaccinated patients with a frequent occurrence of frustrane forms. El'bert and his assistants emphasized, therefore, the great value of anti-tularemia vaccinations not only for the prevention but also for the control of outbreaks.

As will be gathered from the foregoing pages, the initial studies on tularemia immunization with live vaccines led to the introduction of three types of these prophylactics--Gaiskii's fluid virus-vaccine; the fluid egg-yolk vaccine of El'bert, and Faibich's dry NIEG vaccine. As may be conveniently summarized at the present juncture, the practically important properties of these three products, according to Sil'chenko (1960), were as follows:

Gaiskii's fluid virus-vaccine was prepared through dilution of the washings of the attenuated cultures with normal saline so as to obtain a standard of 50 million organisms per ml. The vaccine was administered subcutaneously in a dose of 0.4-0.5 ml (20-25 million organisms). For children Gaiskii manufactured a special vaccine containing 10 million organisms per ml and this was used in doses of up to 1 ml for the age group from 10 to 16 years.

The vaccine produced an immunity of a high degree and long duration. Kazberiuks (1949) established in the latter respect a persistence of positive tularin

Tularemia-II/34

reactions in 82% of a group inoculated with Gaiskii's vaccine for a period of 3 years (limit of observation), while according to Sil'chenko (1952) such tests still gave a positive result in 70.6% of a group of people immunized in this manner 6 years previously. Though many of these persons lived in active tularemia foci, in contrast to the not vaccinated part of the people they remained free from the disease.

However, as noted before, while Gaiskii's vaccine was highly efficacious, its large-scale use in the field was rendered rather difficult by the instability of the product. As shown by the initial experiences of Kosmachevskii (1944), administration of the virus-vaccine in two thirds of the immunized persons led to local and/or general reactions, but as a rule these were slight or at most moderate. Thus only three out of the first group of 50 persons vaccinated under Kosmachevskii's supervision had higher fever (38° - 39°C).

El'bert's egg-yolk vaccine. - Though, as stated by Sil'chenko (1960), the usual method of preparing El'bert's vaccine through cultivation of Gaiskii's strain 15 in Drozhevskina's egg-yolk medium was expedient, it excluded the possibility of accurately determining the bacteriological standard of the products thus obtained.

It was assumed at first that the egg-yolk vaccine could be stored for three months before use, but it was found afterwards that, if kept at room temperature in summer particularly, the vaccine lost its immunogenicity at a considerably earlier date. Therefore a period of storage for not more than two months at a temperature from 4° to 10°C was prescribed afterwards. As has been mentioned already, El'bert's vaccine was administered by the cutaneous route with the aid of the technique adopted for smallpox inoculation.

While admitting the good results obtained with this method by El'bert and his associates (see above), Sil'chenko pointed out that owing to the absence of means of accurately standardizing the egg-yolk tularemia vaccine there were marked inequalities in the degree of the reactions caused by different batches. Still, this vaccine was mainly used for immunization against tularemia in the Soviet Union until in 1950-1951 large-scale advantage could be taken of the dry vaccines.

Faibich's dry NIEF vaccine. - As noted above, the dry vaccine of Faibich and Tamarina was originally prepared from Gaiskii's strain 15, but later on these two workers also incorporated the attenuated strains No. 10 and No. 33. Recently, as also mentioned before, the reconstituted strain 15 and the strain 155 of Emel'ianova (1957 a) are used in the Soviet Union for the manufacture of dry tularemia vaccines.

In agreement with the initial findings of Faibich and Tamarina (see page 29) later observations quoted by Sil'chenko showed that the dry tularemia vaccine remained potent after prolonged periods of storage (2 years at low temperatures and up to 300 days at 18°-20°C). However, the regulations in force at present limit the period of storage of the dry tularemia vaccine at a temperature of 4° to 10°C to one year.

As described by Sil'chenko, at first the NIEG vaccine, after it had been dissolved under sterile precautions with adequate amounts of normal saline, was used in doses of 12.5--25 million organisms for subcutaneous injection. However, from 1946 onwards it was made available for cutaneous inoculation. Initially a bacterial standard of 2 billion organisms was recommended for this purpose, but nowadays, owing to the use of more active strains and the adoption of improved methods of manufacture and assay, a bacterial content of one billion per ml is prescribed.

Concluding his description of the three types of tularemia vaccines, Sil'chenko stated that the NIEG vaccine gave a higher number of takes than the egg-yolk vaccine but that it produced reactions somewhat more frequently than the latter, the more so, as it was used in a higher dose than the egg-yolk vaccine.

Recent investigations

As summarized by Olsuf'ev in a comprehensive report rendered in 1958, within recent years the Soviets have made great progress in further investigations of the fundamental as well as of the practical problems of anti-tularemia immunization with live vaccines.

Most important among the observations of a fundamental nature were the following:

1. As stated in the Olsuf'ev summary, further studies on the antigenic structure of the tularemia bacillus by him and by Emel'ianova (1957) had shown that

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"the cells of the virulent S-culture possess envelope (Vi) and somatic (O)-antigen complexes, but no flagellar (H) antigen. Fully attenuated R strains, bereft of virulence and immunogenicity, possess only an O antigen, practically identical with that of the virulent cultures. The vaccinal SR cultures take, as far as their antigenic structure is concerned, an intermediate position. They have the O antigen and a reduced amount of the Vi antigen. Virulence and immunogenicity of the tularemia bacillus are closely related to this antigen which becomes completely lost in the process of attenuation."

2. Shipitsina and Emel'ianova (1956) established that the above described differences between virulent and avirulent growths were paralleled by differences in the chemical composition of the organisms: biochemically 5 fractions could be found in virulent tularemia bacilli and large amounts of lipoids and protein-polysaccharide substances were present in these organisms. The antigen of the R strains showed only two chemical fractions and was devoid of a considerable part of the protein-polysaccharide compounds and of almost all the lipoids.

As Olsuf'ev added, the antigenic substances obtained from the virulent S organisms with the aid of chemical procedures only partially immunized laboratory animals--a fact proving the lability of the Vi complex. He stressed the importance of improving the methods of isolating these substances so as to obtain a potent chemical vaccine.

3. Earlier studies by Emel'ianova (1953) had led to the practically important as well as theoretically interesting result that marked differences existed between the aspect of the colonies of the tularemia bacillus in the S, SR or the R state, if grown on plates of fish hydrolysate--yeast autolysate agar, to which 0.1% cystine, 1% glucose and 10% defibrinated or citrated rabbit blood¹ had been added (see Emel'ianova 1951, 1958). As summarized by Emel'ianova in her contribution to the work edited by Olsuf'ev and Rudnev (1960), fully virulent tularemia bacilli, if grown on this

1. As stated by Emel'ianova (1960, p. 58), horse blood could be used in place of rabbit blood.

medium, appeared in the form of convex, glistening, smooth, bluish-white, not transparent colonies with a diameter of 1-2 mm, whereas the colonies of weakly virulent strains (or strains of the "immunogenic" type) were larger and whitish. The avirulent strains finally formed transparent, greyish and flattened colonies. Growths of this kind gave a positive tryptaflavin reaction and were not pathogenic when subcutaneously injected into white mice in doses of 100 million to one billion organisms or if administered by the respiratory route in 10 million doses to guinea-pigs. The mice given these enormous doses did not become immune to challenge with even minimal numbers of virulent tularemia bacilli.

4. The studies of Gaiskii on the attenuation of tularemia strains were continued by Maiskii (1948, 1949 a, b) and by Emel'ianova (1949, 1950). As summarized by Olsuf'ev, these observers found that it was possible to attenuate the virulence of any tularemia strain but that the rapidity of the process markedly differed, depending upon the methods used and the individual properties of the strains in question. Olsuf'ev also added that at certain stages of the process of attenuation a selection took place. By this selection more attenuated organisms, because better adapted to a multiplication on artificial media, in the course of repeated subcultivations began to "squeeze out" the less attenuated bacterial cells.

Dealing with the practically most important problem of the selection and maintenance of adequate vaccinal tularemia strains, Olsuf'ev and his associates (1958) summarized that in spite of all care taken Gaiskii's strain 15, after it had served well for about 15 years, began to lose its valuable properties: a process of dissociation, leading to the appearance of non-immunogenic R forms, was apt to take place in the course of vaccine manufacture and hand in hand with this the residual virulence and the immunogenicity of the strain became markedly lowered. It was indispensable, therefore, to reconstitute this strain and it was desirable at the same time to produce other vaccinal strains. Outstanding success in both directions was obtained by Emel'ianova. As described in detail in two accounts published by her in 1957, she had been able to restore the residual virulence and the immunogenicity of Gaiskii's strain with the aid of repeated passages through laboratory animals. At the same time she obtained through serial subcultivation and selection of suitable colonies for further growth a new vaccinal strain designated as No. 155.

Tularemia-II/38

Tests made in cooperation with the Tarasevich State Control Institute (Gosudarstvennyi kontrol'nyi institut or, briefly, GKI) showed that both strains possessed a satisfactorily high residual virulence for white mice and were capable of protecting 98%, respectively 96% of these animals against challenge with 1,000 DCL of a virulent tularemia culture. Subcutaneous administrations of either strain in one-billion doses were innocuous for guinea-pigs and rendered the animals highly immune.

Cutaneous administration of the vaccines prepared from either of the two strains first to volunteers and then to groups of almost 2,000 persons each also gave fully satisfactory results. As established in the course of these campaigns, the vaccines prepared with the new strains proved almost fully potent when used in 50% dilution. Even 1:10 dilutions of the vaccine manufactured from the reconstituted strain 15 still produced 65.8% takes, while the corresponding figure for the vaccine made with the strain 155 was 71%. It is worthy of note that, since the new vaccines had been prepared still with the aid of nutrient media which did not curb dissociation instead of by the technique recommended by Koliaditskaia and Shmurygina (see below), these new vaccines usually did not contain more than 20-40% of immunogenic organisms. Nevertheless, in this respect they compared favorably with the vaccine made from Gaiskii's original strain which contained only 5% of such organisms.

Koliaditskaia and Shmurygina (1960), two of Olsuf'ev's co-workers made great improvements in the manufacture of live tularemia vaccines. These observers established that the methods of manufacture hitherto used, because they involved the use of different kinds of media, rather favored dissociation. Testing each of the media in question separately, the investigations were made on coagulated egg-yolk substrates. In order to take advantage of this fact and to avoid at the same time an excessive use of the latter media, Koliaditskaia and Shmurygina resorted to a procedure thus described by Emel'ianova (1960):

"From ampules containing a dried culture of a vaccinal strain subcultures were made on coagulated egg-yolk slants; from these the growth is transplanted to agar surfaces slanted in flasks (mattresses). To obtain a good yield on these, one uses fish-hydrolysate agar containing cystin and glucose,

on the surface of which a 60% emulsion of egg-yolk in normal saline is poured and this is followed by coagulation of the medium at 80°(c). As shown by Koliaditskaia and Shmurygina, this procedure considerably reduces the dissociation of the vaccinal strain in the course of vaccine manufacture. Transfers from the mattresses are used for growth in semisolid media with the aid of submerged cultivation under aeration."

Emel'ianova added that the semi-solid medium used for this purpose contained 20%-30% of hydrolysate made from fresh fish (or liver or meat), 10% of gelatine hydrolysate, 1%-5% of yeast autolysate, 1.5% gelatine, 0.5% sodium chloride, 1% glucose and 0.1% cystine (pH 7.2-7.3).

According to Olsuf'ev and his associates (1958), the vaccines prepared in the above manner contained at an average 70% of immunogenic organisms and it was possible, therefore, to reduce the bacterial standard of the products to 1 billion per ml. The use of less concentrated vaccines was particularly desirable when dealing with persons who had suffered from tularemia or had been previously vaccinated. To exclude such persons during a mass vaccination campaign was rather difficult.

Olsuf'ev (1960) quoted the now valid instructions for the selection of vaccinal strains and for the manufacture and testing of tularemia vaccines:

"Fully valuable vaccinal strains show the following properties (Emel'ianova, 1957): (They consist of) small gram-negative cocci, somewhat larger than the virulent organisms; they are slimy and stain weakly with all usual stains. The vaccinal culture is well agglutinated by specific sera with formation of a stable sediment which on shaking is broken into large floccules (Vi agglutination). The amount of non-immunogenic organisms in the population should not exceed 20%; the residual virulence for white mice ought to be not lower than 30% and not higher than 50% (these figures indicate the mean percentages of death of mice subcutaneously injected with doses

Tularemia-II/40

from 100 to 1 million organisms determined according to the optical standard of the GKI).¹

Not less than 90% of the surviving mice (ought to be) resistant to subcutaneous infection with 1,000 lethal doses of a highly virulent strain. The vaccinal strains, if subcutaneously administered in one-billion doses, (ought to be) innocuous for guinea-pigs weighing not less than 400 g. Suspensions containing 1 billion organisms per ml, according to the GKI standard if used for the cutaneous inoculation of guinea-pigs (by scarification) ought to produce a local reaction consisting of hyperemia and infiltration of the tissue measuring not less than 0.5 cm.... The skin reaction becomes manifest not later than two days after vaccination.

Cutaneous administration of a suspension made from the dry vaccine, which contains after re-suspension 1 billion of organisms per ml, ought to ensure takes in not less than 24 out of 25 inoculated human subjects. The vaccinal process ought to run mildly, leading to a moderate local skin reaction but, as a rule, not to a general reaction. Within 30 days those vaccinated ought to show a positive tularin reaction and a positive agglutination reaction at a titer of 1:20 or at a higher titer. The dry vaccine currently used for the vaccination of human beings is kept in vacuo in ampules. If stored at temperatures from +4° to +10°(C) it remains fully potent for one year. A massive growth for the production of the vaccine is obtained either on solid media or in

1. In this connection it is important to note the following statement by Emel'ianova (1960, p. 53): "A direct count of the amount of virulent tularemia bacilli and of typhoid bacilli (forming the bacterial standard of the GKI) under the microscope showed that there are 10-12 times more tularemia bacilli than typhoid bacilli in suspensions of the same turbidity (Emel'ianova, 1951). This is due to differences in the size of these two microbial species. Suspensions of tularemia bacilli corresponding in turbidity to the one-billion standard contain per ml not 1 billion but 10-12 billion of separate cells."

fluid media, in the latter case with the aid of the submerged method (under aeration).... An important point in the manufacture of the vaccine is to exclude the appearance of dissociation or to reduce it to a minimum. A simple and fully reliable medium for controlling the quality of the vaccine consists of cultivation on plates with the medium of Emel'ianova. With the aid of this medium one can ascertain the number of live organisms present and the numbers of immunogenic organisms among them. The dry vaccine which is issued should contain after reconstitution not less than 1 billion live organisms per ml with not less than 50% of immunogenic O(Vi) cells."

As may be conveniently added, Faibich (1959), experimenting with white mice, found that the efficacy of tularemia vaccines prepared from his strain No. 10 was increased by the addition of 0.2% agar solution, 1% gelatine solution, 1-5% solutions of gum-arabic, 5% glycerol solution or 5% of a malva decoction. These observations were in accord with those of Zlatkovskii et al. (1947) who had found that the minimal dose of live tularemia vaccines prepared from strains No. 10 and No. 15 capable of producing an allergic reaction in man was 10 times less if an agar solution instead of normal saline was used as vehicle. Faibich stressed that the reactions produced by the subcutaneous administration of tularemia vaccines containing agar, gelatine or gum-arabic in the above mentioned percentages were not increased.

Hand in hand with the above described efforts to improve the quality of the tularemia vaccines, much attention was paid to an improvement of the methods for the evaluation of their efficacy, particularly to the tularin test.

In a short historical account of the use of this test for diagnostic purposes, Berinskaia and Afanas'ev (1941) stated that advantage of this method in the Soviet Union was first taken by Rappoport and Bychkov (1931 - see page 14 of the first part of this review). In 1934-1935 studies of this method were made by Khatenever and his associates (see Khatenever et al., 1935) who recommended as allergen the use of a suspension of tularemia bacilli in normal saline containing 3% glycerol with a standard of 250 million organisms per ml, killed by heating at 65°C for 1/2 hour (see Khatenever, 1941). However, in 1938 the

Tularemia-II/42

standard of the tularin produced in this manner was lowered to 100 million organisms and in this form it was amply used for intracutaneous tests. While the use of this method permitted an early diagnosis of the disease (usually on the 3rd to 5th day of illness), the intracutaneous administration of tularin often led to general as well as to serious local reactions. Moreover, a trained personnel and special syringes and needles were required for the proper performance of such tests.

It was under these circumstances, regrettable as well as surprising, that for many years no attention was paid to the proposal made already in 1934 by Volferz to administer the tularin by the cutaneous (epidermal) route. As this worker described in her original paper, the tularin prepared according to Khatenever's method caused but slight reactions when used in this manner. She resorted, therefore, to a tularin containing 2 billion organisms per ml which was administered in a manner identical with that adopted for smallpox inoculation. While tests of this kind gave negative results in 7 healthy persons, they led to positive local reactions in each of the 12 test persons who had a history of a tularemia attack.

It is of interest that Volferz obtained identical results by the use of a formol-killed tularemia vaccine for the skin tests instead of tularin.

As summarized by Drobinskii (1945), the advantages of cutaneous tularin tests were again pointed out in 1935 by Khatenever, according to whom this method, with rare exceptions, yielded results identical with those of intracutaneous allergic and of agglutination tests. Drobinskii added, without furnishing a reference, that Khatenever and TSvetkova afterwards prepared a special tularin for skin tests with a concentration of 1 billion organisms per ml. When applied cutaneously, this tularin produced in 105 tularemia patients reactions analogous to those following intracutaneous tests. Similarly Shmutter, whom Drobinskii also quoted, found that only 8 out of 181 tularemia patients did not react identically to cutaneous and intracutaneous skin tests and was inclined to ascribe these divergent results to technical shortcomings or misreading of the results rather than to an inferiority of the cutaneous method.

Berinskaia and Afanas'ev (1941) made simultaneous use of the intracutaneous and cutaneous administration of tularin to (a) 31 tularemia patients ill for from 7 to 45

days; (b) 3 persons who had contracted the infection from 3 months to 6 years previously; (c) 20 controls (6 healthy persons and 14 suffering from other diseases). The tularin used for the skin tests had a standard of 2 billion organisms and was inactivated by heating it 3 times for one hour at 70°C. Side by side with the tularin tests the sera of the first two groups were used for agglutination tests (which invariably gave a positive result).

Summarizing the results of the tularin tests, the two authors stated that

1. The cutaneous allergic reaction with tularin is fully specific and like the intracutaneous test is suitable for the early diagnosis of tularemia.

2. Both tests remain positive for many years (according to our observations for 3-6 years) and are thus suitable for a retrospective diagnosis of tularemia.

3. While diagnostically as valuable as the intracutaneous method is preferable because it produces milder reactions and is easier to perform.

However, Drobinskii (1945), making simultaneous use of cutaneous and intracutaneous tularin tests and of agglutination tests in a group of tularemia patients the size of which he did not state, maintained that

"The best method of the immunobiological diagnosis of tularemia remains the serological-allergic method with the use in the first line of the intracutaneous tularin test, particularly in the early stage of the disease. The use of the cutaneous tularin test alone, though it is technically the most simple method, does not permit the detection of a considerable part of the persons who suffer or have suffered from tularemia."

It was no doubt because of contentions like that of Drobinskii that the early recommendations for the performance of cutaneous tularin tests were generally disregarded and the intracutaneous method was preferred at first to test besides patients and convalescents also groups of people who had been vaccinated against tularemia.

Tularemia-II/44

Dealing with the history of the use of the tularin test for the latter purpose, Sil'chenko (1960) stated that

"B. IA. El'bert and N. A. Gaiskii (1941), M. M. Faibich and T. S. Tamarina (1946), I. N. Maiskii (1953) Olsuf'ev (1953) and others concluded from numerous experiments in laboratory animals and supplementary observations in man that an intense immunity against tularemia infection is produced in the body not only through an attack of the disease but also through immunization with live tularemia vaccine and is manifested by the outcome of tularin and agglutination tests. Gaiskii (1944) demonstrated that the phenomenon of allergy constitutes the fundamental indicator of immunity and also a criterion of the potency of the vaccine, the adequacy of its dosage and the efficacy of the inoculations. B. IA. El'bert (1946), on account of the fact that the tularin test is an indicator of a past tularemia attack and at the same time of a stable and long-lasting immunity, utilized this test for an assessment of the specific protection of the vaccinated against tularemia. He obtained positive results with the tularin test in a majority of the vaccinated persons as well as in those recovered from tularemia. M. M. Faibich and T. S. Tamarina observed that the live tularemia vaccine produced in the inoculated the appearance of allergic and agglutination reactions. They found that the time of the appearance of the allergic reaction in persons vaccinated with dry tularemia vaccine coincided with that of the manifestation of this reaction in tularemia patients. These findings enabled N. A. Gaiskii, B. IA. El'bert, M. M. Faibich and afterwards other workers widely to use the immunological reactions (in the first line the tularin test) for a determination of the inoculability as well for a detection of the allergic state and the preservation of the immunity in the vaccinated."

Taking early advantage of this method, Chernina (1950) reported that the use of intracutaneous tularin test in the case of 1,657 persons who had been vaccinated with tularemia egg-yolk vaccine, gave positive results 3 months after

the immunization in 100%, after 6 months in 94-98%, after one year in 10-31%, but proved negative 15 months after the vaccination. Agglutination tests made at the same intervals proved 100% positive after 3 months, at titers of 1:800 in 37%, at 1:400 in the others. All those vaccinated still reacted positively after 6 months at titers of 1:100-1:400, after 9 and 12 months at titers of 1:50 or 1:100 (15%). Agglutination tests made 15 months after the vaccination gave negative results.

Resorting to intracutaneous tularin tests for the purposes of an epidemiological survey, Vasil'eva and her associates (1952) stated that they had used this method for the examination of 405 persons who had been vaccinated with Gaiskii's vaccine. Out of the 173 of these persons who reacted positively to tularin, only a minority (8.6%) showed as marked reactions as the majority of individuals suffering from tularemia or having recently recovered from it. While the intensity of the reactions was not influenced by the length of time which had elapsed since the vaccination, it was found that the number of positive reactors incessantly decreased in proportion to the length of this interval, 71% proving positive when tested one month after immunization, 50% after 6 months and only 10% after one year--a figure which, as the editor maintained in a footnote, was quite unusually low. In fact, Vasil'eva and her co-workers themselves reported quite different results in a note published in 1953, in which they recorded observations in (a) vaccinated persons and (b) such who had been re-vaccinated one year after the initial immunization. (The size of these two groups is not stated.) The percentage of positive tularin reactions in the two groups was as follows:

<u>Group</u>	<u>Time After Vaccination or Re-vaccination</u>			
	<u>2 Months</u>	<u>6 Months</u>	<u>1 Year</u>	<u>2 Years</u>
Vaccinated	90.4	81.3	79.6	74.0
Re-vaccinated	91.8	-	91.3	-

The authors added that

"The maximal percentage of marked (17.9%) and moderately marked (62.8%) reactions was observed 6 months after vaccination; after one year 50.1% of the reactions were moderately marked; 2 years after vaccination 65% of the reactions were slight and marked reactions were quite absent."

Tularemia-II/46

In 28 out of 30 persons with a positive tularin reaction tested two months after vaccination with tularemia antigen, the agglutination test was positive at titers not exceeding 1:50 or 1:100.

In the re-vaccinated the percentages of marked or of moderate reactions to tularin were 69.5% after two months and 65.1% after one year. Agglutination tests made two months after re-vaccination in 22 persons positively reacting to tularin invariably gave a positive result, in 63% at titer of 1:200 to 1:400. The efficacy of re-vaccination was thus evident.

While during the observation period sporadic tularemia attacks were recorded among the not immunized part of the population, the vaccinated and re-vaccinated groups, though exposed to the same risks of infection, remained free from the disease.

The workers contributing to the report on the efficacy of anti-tularemia vaccination edited by Olsuf'ev (1953), to which reference will be made later in this review, invariably resorted to intracutaneous tularin tests. The same procedure was still used by Martinevskii (1957) when examining 63 persons who had been vaccinated 4 years previously with NIEG vaccine. Of this group 51 members still reacted positively, 24 of them showing most marked reactions. Agglutination tests with the sera of 48 of the vaccinated, taken immediately before the administration of tularin, gave positive results in 38 instances at titers ranging from 1:10 to 1:160.

Summarizing the results of observations on the intracutaneous administration of tularin, Sil'chenko (1960) pointed out that some workers had recommended making these tests not with the usual dose of 0.1 ml but with larger doses. However, noting not only that tularin, if used in 0.1 ml amounts, gave clear-cut results but also that even this dose not rarely produced untoward severe local and general reactions, Sil'chenko took a firm stand against the use of increased dosages for the intracutaneous tularin tests. He pointed out in this connection that according to data recorded by Maiskii (1953), intracutaneous tularin tests, made in the usual manner 9 months after vaccination, had led to necroses at the site of injection in 10% of the test persons, in 3% to shivering and fever up to 38°C, in 14% to a general indisposition and headache, in 29% to an involvement of the regional lymph nodes. It was because

of these observations that Popov and his associates (1953¹, 1958) and then Olsuf'ev and co-workers (1955, 1956, 1958) again paid attention to the advantages of the cutaneous method of tularin administration. Most important among the investigations made in this direction were large-scale tests made in 1955 by Olsuf'ev and numerous co-workers in various regions of the European part of the Soviet Union and in West Siberia which according their 1956 publication led to the following conclusions:

- "1. Cutaneous tularin tests made by 8 anti-tularemia stations on 3,968 persons who had been vaccinated against tularemia and on 212 persons who had suffered from this disease in the past, fully demonstrated the suitability of this method for the detection of an allergic state in these groups of people.
2. In mass examinations of the population cutaneous tularin tests proved to be fully specific and much easier to apply than the intracutaneous tests.
3. Cutaneous tularin tests are fully suitable for mass examinations undertaken for the detection of the tularemia-immune stratum of the population living in natural foci of the infection; in particular it is not difficult with the aid of such tests to detect persons with a positive (specific) allergic reaction if no records are available to show up those who had been vaccinated or had suffered from the disease.
4. Cutaneous tularemia tests are less reactogenic than those made by the intracutaneous route and produce more rarely than the latter side effects in the vaccinated or in persons who had suffered from the disease.
5. Cutaneous tularemia tests give clear results in persons who had been vaccinated or had suffered from the disease long ago (8-10 years).
6. Comparative tests made by the cutaneous and intracutaneous routes gave almost fully identical results.

1. It was not possible to find a reference of the 1953 publication of Popov et al.

Tularemia-II/48

7. Tularin prepared from a vaccinal strain is as allergenic as that made from a virulent strain.

8. Tularin in a concentration of 2 billion per ml in special tests gave a somewhat higher percentage of diagnostic reactions than preparations with a concentration of 1.5 billion. The higher concentration led (only) to an inconsiderable increase of the side effects and the reactions themselves were fully tolerable.

9. For the manufacture of tularin for cutaneous tests it is possible to use a vaccinal strain and to adopt a bacterial standard of 2 billion organisms per ml.

10. Considering the simplicity and easy availability of the technique necessary for the large-scale use of cutaneous tularin tests, their high efficacy for the determination of the allergic reaction in man and their not high reactogenicity one may postulate the use of this method individually as well as for mass examinations of the population (to detect the immune stratum, to determine the efficacy of the vaccinations and the necessity for re-vaccination)."

In fact, as stated by Sil'chenko, a tularin prepared for cutaneous administration in the Gamaleia Institute of Epidemiology and Microbiology is now amply used in the Soviet Union.¹

It may be conveniently added that Uglovoi, in a paper published in 1963, stated that tularins, prepared for cutaneous tests from Gaiskii's strain No. 15 and from the same reconstituted strain remained allergenic when stored at temperatures ranging from 4° to 20°C for periods up to 5 years and 7 months (limit of observation). Untoward

1. Besides those mentioned in the text, articles recommending the cutaneous administration of tularin in preference to the intracutaneous route have been published also by Borodin & Koroleva (1955), Belostotskaia et al. (1957), Vasil'eva & Kyrchnikov (1959) and Timofeev & Andronnikov (1962).

reactions produced by one of the tularin series tested, which accidentally had a bacterial standard of 2.4 billion instead of 2 billion, showed that the latter concentration should not be exceeded.

According to Sil'chenko's summary, Kazberiuik and his associates as well as Martinevskii in 1956 had proposed for the determination of the allergic state to resort to the administration of one drop of live tularemia vaccine instead of tularin. In support of this proposal the authors claimed not only that the live vaccine caused less side effects than intracutaneously administered tularin and was a better allergen than the latter but also maintained that by using this method one was apt to produce an immunity against tularemia or to enhance it. Sil'chenko stated that

"This proposal was not accepted, mainly because the persons tested are apt to include people who had had tularemia and thus showed an increased reactivity to the administration of the live vaccine (Sil'chenko, 1957; Olsuf'ev, 1958). On the other hand, the administration of half a vaccine dose (one drop) cannot produce a full immunity in non-immune persons, thus creating a need for their re-vaccination at intervals shorter than 5 years.... After the approbation and adoption of the cutaneous tularin administration, which causes almost no side effects in vaccinated persons and produces little marked reactions in people who had had tularemia, it is impossible to accept the proposals of Kazberiuik and Martsinevskii to use live tularemia vaccine as allergen."

It is interesting that a few workers recommended the use of other means instead of tularin antigenic fractions of the tularemia bacillus for the allergic tests. It first deserves attention in this respect that Maiskii and Shipitsina (1952 a, b, c) recommended for the rapid diagnosis of tularemia a preparation called tuallergen which, as stated by Uglovoi and his associates (1962), consisted of the antigenic complex of the tularemia bacillus freed from lipides. Summarizing the results they had obtained with this compound in 239 persons (12 tularemia patients; 19 who had suffered from this disease in the past; 31 persons vaccinated against tularemia; 16 brucellosis patients; 77 patients with other

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infectious diseases and 84 healthy individuals), Maiskii and Shipitsina (1952 c) stated that it was preferable to tularin because (1) it produced an allergic reaction within 5-20 minutes as against 24-48 hours in the case of tularin and (2) as a rule the administration of tuallergen did not lead to an increase of the body temperature, enlargement of the lymph nodes and necroses at the site of injection. As a rule the tuallergen did not give positive reactions in patients suffering from infectious diseases other than tularemia. False positives were observed only in 4.5% of the persons tested, mainly in such who suffered from brucellosis or had been affected by this disease in the past. Thus, the authors claimed, tuallergen was apt to be helpful for a quick assessment of the allergic condition of vaccinated persons as well as of such who had suffered from tularemia. It could be useful also when selecting personnel for activities involving a risk of tularemia infection, e. g. contingents of hunters or threshers.

Recently Uglovoi, Savel'eva and Shipitsina (1962) recommended for the allergy tests the use of a modified product, called tuallergen-2, which was prepared from a vaccinal tularemia strain with trichloroacetic acid according to Boivin's method. Results obtained in preliminary trials through intracutaneous administration of this preparation were as follows:

<u>Antigen Dilu- tions Used</u>	<u>Results in Persons Who Had Tularemia at</u>		<u>Vaccinated</u>		<u>Re-vaccinated</u>		<u>Controls</u>	
	<u>Least 15 Years Ago</u>							
	<u>Positive</u>	<u>Negative</u>	<u>+</u>	<u>-ve</u>	<u>+</u>	<u>-ve</u>	<u>+</u>	<u>-ve</u>
1:50,000	6	0	7	0	0	8	0	16
1:10,000	Not tested				8	0	0	16

Actual use of tuallergen-2 (in a dilution of 1:25,000) was made during a vector-borne outbreak taking place in 1960 in the Iakutsk ASSR, when positive results were obtained within 30-45 minutes in all but one of 39 patients who had fallen ill with tularemia 4-56 days before. Cutaneous tularin testes proved positive in all the patients.

Results obtained with tuallergen-2 and tularin in 60 persons who had been vaccinated against tularemia 2 years ago, were thus tabulated by the authors:

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<u>Persons Tested</u>	<u>Dilutions Used</u>	<u>Tuallergen-2^a</u>			<u>Tularin^c</u>	
		<u>Positive</u>	<u>Negative</u>	<u>Doubtful</u>	<u>Positive</u>	<u>Negative</u>
34	1:25,000	23	7	4 ^b	26	8
26	1:50,000	18	8	-	19	7

Remarks: (a) Read after 1 hour. (b) In 3 of these instances the reaction became positive within a day. (c) Read after 48 hours.

The authors noted that Kontorina (1958) had obtained results analogous to those of tularin when resorting to the cutaneous administration of a polysaccharide fraction of the tularemia bacillus obtained with acetic acid according to White's method and used in a dilution of 1:100. They decided therefore to make analogous tests with a tuallergen prepared in the same manner on 19 persons who had been vaccinated against tularemia 2 years ago. Results were as follows:

<u>Time of Reading (Hours)</u>	<u>White's Antigen(1:100)</u>			<u>T u l a r i n</u>		
	<u>Positive</u>	<u>Negative</u>	<u>Doubtful</u>	<u>Positive</u>	<u>Negative</u>	<u>Doubtful</u>
3	7	11	1	1	17	1
24	17	2	-	13	5	1
48	15	4	-	17	2	-

The conclusions reached by the authors were that tuallergen-2 was a product suitable for the rapid diagnosis of tularemia but that, before it could be used for ascertaining the state of immunity in vaccinated groups of the population, tests on a large-scale were necessary so as to determine the activity of different concentrations of this allergen and the time suitable for the reading of the results.

It has to be mentioned also that two Polish authors, Parnas and Lazuga (1957) recommended in a brief note a

Tularemia-II/52

"Tularin M," obtained from a tularemia strain in the S-phase with the aid of ultrasonic waves, for diagnostic purposes and vaccinotherapy.¹

To complete the present disquisition, attention has to be paid to (1) attempts to resort to the administration of tularemia vaccines by other routes instead of the usual methods of immunization and (2) proposals to combined vaccination against tularemia with vaccination against other infectious diseases.

Vaccination by unconventional routes

- (a) Intranasal vaccination. - Experimental studies on the possibility of producing an immunity against tularemia through vaccine administration into the nostrils have been made by El'bert and his associates (see Puchkova, 1948; El'bert, 1952; Kirvel', 1953; El'bert et al., 1954 a, b). Evaluating this method in comparison with cutaneous vaccination, El'bert, Iudenich, Kirvel' and others (1954 a) briefly referred to good results obtained in 1952 with the intranasal administration of dry or egg-yolk tularemia vaccines to musk-rats and reported more exhaustively on identical experiments made on 149 guinea-pigs. In contrast to Kirvel' (1953) who, as quoted by Olsuf'ev (1960), feared that the intranasal application of tularemia vaccine might lead to inflammatory processes in the lymph nodes of the faucial ring or the thorax, El'bert and his co-workers declared that this method of vaccination was innocuous as well as effective. The animals thus protected were found to resist challenge made one month after a single intranasal vaccine administration with 1,000 to 10,000 DLM of a virulent tularemia strain and even dilutions of the egg-yolk vaccine ranging from 1:10 to 1:10,000, intranasally applied in a dose of 2-3 drops, conferred protection against challenge with 1,000 DLM. When sacrificed one month after challenge, only the guinea-pigs immunized intranasally with 1:10,000 dilutions of the egg-yolk vaccine showed signs of tularemia infection in their internal organs.

In a further series of tests, guinea-pigs which had been vaccinated against tularemia either intranasally

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1. A 1955 paper by Tatomir, referring to the preparation of a tularemia antigen free from microbial cells and ballast proteins could not be consulted.

or cutaneously, were challenged with 1,000 DLM at intervals ranging from 3 days to 11 1/2 months after immunization. It was found that 50% of the animals immunized by either method survived challenge when tested 3-5 days later and that protection had become complete in the animals challenged one week after immunization. When tested after 11 1/2 months, still 75% of the vaccinated animals survived.

Agglutinins began to appear in the sera of the vaccinated animals at the end of the first or the beginning of the second week after immunization and reached their highest titer (1:160 to 1:320) after 3-4 weeks. Tularin tests began to give positive results simultaneously with the appearance of agglutinins and still proved positive one year after vaccination (limit of observation).

- (b) Oral vaccination. - As briefly stated by El'bert and his associates (1954 b), administration of tularemia vaccine to musk-rats or guinea-pigs by the oral route produced worse results than intranasal vaccination or immunization through the respiratory tract.

According to Olsuf'ev (1960), Mikhailov (1952) found that in order to obtain success with the "alimentary" method of tularemia vaccination it was necessary to use much higher vaccine doses than those needed for subcutaneous immunization. Still, even oral administration of 100 million doses of tularemia bacilli conferred protection to only 90% of the test animals--a result explained by the bactericidal action of the gastric juice (see Savel'eva, 1956).

- (c) Vaccination by the respiratory route. - In note just mentioned El'bert and his associates recorded that vaccination "through the respiratory tract (dispersion in a chamber)" gave results comparable to those of intranasal inoculation in musk-rats and guinea-pigs. Referring to further experiences with immunization through the respiratory tract, Olsuf'ev stated that

"In tests by R. A. Savel'eva and G. P. Uglovoi on guinea-pigs was shown the possibility of producing an immunity in animals by the introduction of tularemia vaccines through the

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respiratory tract (dispersion of a suspension in the form of a fog in a special chamber). In this one could note a considerable sensitivity of the guinea-pigs to the introduction of the vaccine into the lungs--a dose of 10 million organisms proved lethal for a considerable part of the test animals which succumbed with signs of a total pneumonia (red hepatization of almost all lobes of the lung), the specificity of this process being demonstrated by the isolation of the vaccinal strain in cultures. The guinea-pigs tolerated respiratory administration of 100,000 organisms but had fever for 3-4 days and did not increase in weight during that time."

Results of challenge tests with a virulent strain made 1-2 months after respiratory immunization by different routes were as follows:

<u>Organisms Used for Challenge</u>	<u>Subcutaneously</u>		<u>Method of Challenge</u>			
	<u>Number Chal- lenged</u>	<u>Died of Tula- remia</u>	<u>Intracutaneously</u>		<u>By Inhalation</u>	
			<u>Number Chal- lenged</u>	<u>Died of Tula- remia</u>	<u>Number Chal- lenged</u>	<u>Died of Tula- remia</u>
100	11	1	12	0	13	1
10,000	11	1	12	1	8	5
1 million	9	1	10	0	12	11
	31	3	34	1	33	17

While pointing out that thus the test animals were insufficiently protected against challenge by the respiratory route, Olsuf'ev added that

"In other experiments by R. A. Savel'eva and G. P. Uglovoi it was noted that guinea-pigs vaccinated by the lung route were about as resistant to challenge with a virulent culture by inhalation as the animals vaccinated subcutaneously or intracutaneously."

Evaluating the three above discussed modes of tularemia immunization, Olsuf'ev maintained that

"None of these methods of vaccination has a higher immunisatory efficacy than the cutaneous method and the oral method is even less effective. Vaccination through the respiratory tract proved to be dangerous in the case of an overdosage of the vaccine and tedious to use. The nasal method as well cannot be considered as harmless if one considers how easily the instillation of the vaccine into the nose may lead to an overdosage.... An essential shortcoming of all three methods considered by us is also the impossibility of a (direct) observation of the results. Thus, out of all possible modes of vaccination against tularemia the cutaneous method, proposed by B. IA. El'bert, is practically the best and not dangerous to use, and it is moreover highly efficacious in respect to the various modes of infection under the usual epidemiological conditions."

The fact that Aleksandrov, Gefen and their associates in several publications recently advocated the cause of aerosol immunization against tularemia merits attention. Their statements may thus be summarized:

Dealing with the "Method of aerogenous (inhalatory) immunization and the possibilities of its improvement" in a general manner, Aleksandrov and Gefen (1958 a) stated that in order to obtain dry vaccines in dust form for their work they used inter alia Gaiskii's strain No. 15 (apparently in its reconstituted form), but gave no details of their method of vaccine manufacture. They emphasized the advantages of mass "spontaneous" aerogenous immunization, as being

"most physiological, deep, complete, little traumatic and least labor-consuming, permitting in the shortest time and with a small amount of means and labor to reach large contingents. In this lies the fundamental and most important advantage of the method of aerogenous immunization in comparison with other methods."

The method of individual aerogenous immunization, on the contrary, offered in the opinion of the two authors no advantages over the other modes of vaccination.

Tularemia-II/56

Aleksandrov and Gefen, described their use of the aerogenous immunization of small laboratory animals (which, however, because of their shallow respiration and the anatomical and physiological peculiarities of their respiratory tract were rather unsuitable for such tests) a chamber measuring 1 m³, in which it was possible to experiment simultaneously on maximally 50 white mice, 10 guinea-pigs or 3 rabbits. Sheep were immunized in chambers with a capacity of 3 m³, in which 10 animals could be accommodated at one time. As established by a series of special tests with the vaccinal tularemia and brucellosis strains, only vaccines which produced growth on suitable media in dilutions of not less than 10⁻⁸ were suitable for aerogenous immunization.

In order to test the innocuousness of the dry tularemia and brucellosis vaccines, various doses were administered to white mice and guinea-pigs either intranasally or subcutaneously. It was found that the reactions produced by the former (intranasal) administration of these vaccines were somewhat slighter than those caused by subcutaneous injection.

For inhalation tests groups of white mice or guinea-pigs were exposed to the action of the vaccines for 20-30 minutes and then housed in ordinary cages for observation. Findings in animals sacrificed at various intervals indicated that the vaccinal strain 15 could be isolated from their organs from 5 to 25 days after immunization. Results of agglutination and tularin tests in the aerogenically immunized guinea-pigs and in subcutaneously vaccinated controls were as follows:

<u>Mode of Vaccination</u>	<u>Vaccine Dose</u>	<u>Agglutination</u>		<u>Titers</u>	<u>Percentage of Positive Tularin Tests 90 Days After Immunization</u>
		<u>15-30 Days</u>	<u>30-60 Days</u>	<u>60-90 Days</u>	
Aerogenous	Dispersion of 4 g of the vaccine	1:50	1:100	1:50	100%
Subcutaneous	20 million organisms	1:50	1:100	1:50	100%

Aerogenous vaccination thus gave results identical with those produced by subcutaneous vaccine administration.

In a second communication (1958 b) appearing soon after the publication of the above quoted article, Aleksandrov,

Tularemia-II/57

Gefen and their associates reported in somewhat greater detail on aerogenous immunization against some zoonoses including, besides plague, brucellosis and anthrax, also tularemia. They used for tests on small laboratory animals chambers with a capacity of 0.5-1.5 m³. Immunization of these animals was effected

"by spraying 3-10 g of the aerogenous vaccines, containing in 1 g tens or hundreds billions of live organisms. Exposition to the vaccines lasted 30-60 minutes. During that time the animals inhaled from some hundred thousands to tens of millions live microbes."

Guinea-pigs immunized in this manner and one month afterwards challenged by the subcutaneous route or by inhalation with a virulent tularemia strain in doses of 100 to 1,000 DLM showed a survival rate of 67-80% as against 88-100% in the case of animals injected subcutaneously with live tularemia vaccine.

Though, for the reasons stated above, the experimental results obtained with the aerogenous method were not as satisfactory as those of subcutaneous vaccination, the former mode of vaccination was used for a group of human volunteers. For this purpose advantage was taken of special boxes with a capacity of 3.8 m³ or 10 m³ or of a tent with an air volume of 12 m³, the vaccine being distributed in each case with a sprayer. The volunteers were exposed to the action of the vaccine for 15 minutes during which samples were taken so as to determine the bacterial concentration per liter of air.

Out of the 128 persons immunized by the aerogenous method with an estimated amount of 750,000 live tularemia bacilli, a general reaction of moderate severity was noted in two, one of these individuals also showing signs of a local reaction in the form of a quickly passing bronchitis.

An immunological response to the aerogenous vaccination began to become manifest already after one week. During the following three weeks the agglutination titers rose to a maximum of 1:2,560 and tularin tests gave a positive result in 96% of the immunized persons. The agglutinin titers in cutaneously inoculated controls were found to be somewhat lower.

In the introduction to a 1960 report on the experimental efficacy of aerosol immunization with dry pulverized

Tularemia-II/58

vaccines against anthrax, brucellosis, plague and tularemia¹ Aleksandrov, Gefen, Gavin and Gapochko thus referred to the technical aspects of their work:

"The dry pulverized vaccines, destined for aerosol immunization, were prepared according to an original method with the following live vaccinal strains: Anthrax STI 1 and 3, Br. abortus bovis 19/BA, Tularemia No. 15 (reconstituted) and Plague 1 and 17. These vaccines had the form of easily dispersible powders of a polydisperse fractional composition.

The efficacy of aerosol immunization with these vaccines was studied on guinea-pigs, rabbits, sheep and monkeys. The small laboratory animals were vaccinated in chambers with an air volume of 1.5-5 m³, the sheep and monkeys in rooms with an air content of 5-20 m³. Immunization was effected by spraying a predetermined amount of the dry vaccines, containing in 1 g from some tens to 1,000-2,500 billions of live organisms. Exposure to the vaccination lasted from 15 to 60 minutes.

In order to determine the vaccinal doses inhaled by the animals, we made determinations of the amount of live organisms per liter of air of the chambers or rooms where the work was done.

These samples were taken with the aid of adsorption apparatus filled with normal saline and connected with an exhaust system. The amount of aerosol taken in by each

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1. This is the third of a series of articles dealing with "Aerosol immunization with dry live vaccines and anatoxins." The first article with the sub-title "Theoretical and experimental premises for working out a method of aerosol vaccination" appeared in the June 1960 number of the Zhurnal mikrobiologii... the second, dealing with a "Study of the efficacy of the aerosol method of immunization and re-immunization with dry pulverized diphtheria anatoxin," in the July number of the same journal.

adsorbing apparatus was controlled with the aid of a rheometer. A determination of the biological concentration of the aerosols was made through titration of the fluids in the adsorbing apparatus by the method of growing 0.1 ml amounts of serial ten-fold dilutions on pairs of solid media....

The inhaled doses of the vaccines were determined with the aid of the formula $AD = \frac{S \cdot T \cdot Vt}{1000}$, where AD was the inhaled amount of live organisms, S the number of organisms in 1 liter of the aerosol, T the time of exposure to the vaccination in minutes and Vt the volume of air inhaled by the test animals per minute."

In the case of tularemia experiments were made in the above described manner on 96 guinea-pigs. The immunologic and allergic responses to the vaccination are shown in the following table:

<u>Time After Immunization</u>	<u>Agglutination Tests</u>	<u>Tularin Tests</u>
	<u>Percentage of Positive Reactions</u>	<u>Percentage of Positive Reactions</u>
15 days	83.0	0
1 month	100.0	73.0
2 months	9.0	30.0
3 months	0	0

Challenge tests made by the subcutaneous route one month after immunization gave the following results:

<u>Challenge Dose (DLM)</u>	<u>Aerosol Immunization</u>				<u>Controls</u>			
	<u>With 100,000 Organisms</u>		<u>With 10 million Organisms</u>		<u>Cutaneously Vaccinated</u>		<u>Normal</u>	
	<u>Number Tested</u>	<u>Died</u>	<u>Number Tested</u>	<u>Died</u>	<u>Number Tested</u>	<u>Died</u>	<u>Number Tested</u>	<u>Died</u>
100	11	5	10	1	5	0	5	5
1,000	9	4	10	2	5	0	5	5
<u>Totals</u>	20	9	20	3	10	0	10	10

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Commenting on these findings, the authors admitted that, as far as these tests went, aerosol immunization gave somewhat worse results than cutaneous vaccination.

In a further communication appearing in December 1960 Aleksandrov and his associates recorded the bacteriological findings in animals which after aerosol immunization with the vaccines studied by them had been sacrificed at various intervals. Summarizing the results obtained in this manner in 35 guinea-pigs immunized against tularemia, the authors stated that the presence of an initial non-sterile phase of immunity was indicated by the isolation of the vaccinal organisms from the respiratory tract and the adjacent lymph nodes as well as from the blood, spleen and liver in the case of animals sacrificed up to the 15th day. One month after immunization most test animals had evidently reached the sterile of immunity, as only in two out of 11 instances positive cultures could still be obtained from the regional lymph nodes alone, while the cultivations from the respiratory tract as well as from the blood and the parenchymatous organs gave invariably a negative result.

Studying the morbid changes in the organs of animals which had been subjected to aerosol immunization, Gordon and Gefen (1961) noted in the case of the guinea-pigs protected against tularemia

"the appearance of epitheloid and lymphoid cell granulomata in the lymph nodes, the spleen and sometimes the liver, the frequency of which was directly related to the vaccine doses used."

As a rule these granulomata did not undergo necrosis but disappeared without trace or, in rare cases, left scars in the connective tissue.

The statements made in regard to tularemia in the sixth article of their series of publications by Aleksandrov and associates, entitled "Study of the post-vaccinal reactions and immunological efficacy of aerosol immunization with pulverized vaccines (against brucellosis, tularemia, anthrax and plague) in man" (1961) may thus be summarized:

- (a) The relation between the reactions produced in man through aerosol immunization with the reconstituted tularemia strain No. 15 and the sizes of the doses used is shown in the following table:

Tularemia-II/61

<u>Vaccine Doses</u>	<u>Number of</u>	<u>General Reactions</u>				<u>Local Re-</u>
<u>Inhaled</u>	<u>Vaccinated</u>	<u>Total</u>	<u>Slight</u>	<u>Moderate</u>	<u>Severe</u>	<u>action (La-</u>
20 million	82	9	7	2	0	8
50-100 -"-	38	9	8	0	1	4
200 -"-	39	13	12	0	1	8
<u>Totals</u>	159	31	27	2	2	20
		(10%)	(16%)	(1.2%)	(1.2%)	(12%)

Thus even high doses caused as a rule no severe reactions.

- (a) The immunological efficacy of aerosol immunization against tularemia, as manifested by the results of tularin and agglutination tests, was found to be as follows:

<u>Time of Obser-</u> <u>vation (Days)</u>	<u>Tularin Tests</u>		<u>Agglutination Tests</u>		
	<u>Number Tested</u>	<u>Positive</u>	<u>Number Tested</u>	<u>Positive</u>	<u>Mean Titer</u>
7	7	0	19	8	1:2.6
15	42	34	42	27	1:24
30	50	50	50	48	1:319
90	25	19	87	68	1:343
180	61	41	56	55	1:165
240	107	93	-	-	-

The general conclusion reached by the authors was that

"Inasmuch as the doses of the pulverized vaccine within the range of 100 to 200 million, while causing inconsiderable reactions, ensured a marked immunological response (Umstimmung) in the body of the vaccinated, we came to the conclusion that these doses can be considered as optimal for aerosol immunization against tularemia."

Tularemia-II/62

Combined vaccination against tularemia and other infectious diseases

- (1) Tularemia and smallpox. - The combination of vaccinations against tularemia and smallpox deserves primary mention because, having been tried as early as 1945 by El'bert, it was apparently the first of various methods recommended in the Soviet Union for an association of tularemia immunization with immunization against other infectious diseases. As El'bert briefly stated in this connection,

"In a third series of observations on guinea-pigs studies were made on the possibility of a simultaneous and single vaccination with the tularemia virus-vaccine and the smallpox vaccine. At different intervals of time the vaccinated guinea-pigs were tested by cutaneous infection with a virulent tularemia culture and the rubbing-in of smallpox vaccine (while all the controls died) and showed a positive allergic reaction to the vaccinia virus according to Gins."

Akimenko (1949), studying the possibilities of a combined immunization against tularemia and other infectious processes in a more comprehensive manner, resorted inter alia to combined vaccinations of guinea-pigs and rabbits against tularemia and smallpox. According to his brief statements he tested the immunity engendered in these animals against tularemia in the usual manner with tularin, agglutination and challenge tests. In order to establish the presence of an immunity against smallpox he resorted to

"the allergic method (skin tests according to Gins) and the so-called fluorescein test to establish the presence of a generalization of the smallpox virus vaccine in the immune body."

The results obtained by Akimenko with a combined vaccination against tularemia and smallpox were as satisfactory as those with the other combined methods of immunization which he tried and to which reference will be made below. The observations of Arslanova and her associates (1960) who synchronized the administration of tularemia and smallpox inoculation with the administration of other vaccines, will likewise be dealt with in a later section of this review.

It is important to add that according to Klets and his co-workers (1959 b) an instruction issued by the USSR Ministry of Health in 1957 sanctioned the simultaneous vaccination against tularemia, smallpox and brucellosis "in the case of urgent demand."

- (2) Tularemia and brucellosis. - The possibility of combining the immunization against tularemia and brucellosis seems to have been explored first by P'ilipenko and Poliakova, who recorded the results of their experimental work (begun in 1953) in two articles published in 1955 and followed in 1956 by a communication written in association with Shchekina.

In their initial report Pilipenko and Poliakova recorded observations on (a) 16 guinea-pigs injected subcutaneously at the same time but in two different places with 20 million doses of live tularemia vaccine and 1 billion doses of live Brucella abortus BA vaccine and (b) 18 guinea-pigs which received simultaneously cutaneous inoculations against tularemia and subcutaneous administrations of the brucellosis vaccine. Animals of a control group were subcutaneously injected with either the tularemia or the brucellosis vaccine.

The conclusions reached through these experiments were that

"1. Administration of tularemia vaccine to guinea-pigs either subcutaneously or cutaneously together with the subcutaneous administration of brucellosis vaccine led to an immunobiological transformation in the body of these animals. The latter was manifested by an increase of the agglutinin titers in the serum (of the animals) and allergic reactions to the tularin and brucellosis antigens.

These findings show that the causative organisms of tularemia and brucellosis, when being together in the body of guinea-pigs, do not exert an inhibitory action on each other.

2. The immunobiological transformation in the animals receiving this combined vaccination took place in a manner almost identical with that in the guinea-pigs immunized against either of these infections.

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3. The degree of the immunity against tularemia and brucellosis in the combinedly vaccinated guinea-pigs was identical with that in the animals immunized against either infection."

In their second publication Pilipenko and Poliakova recorded the findings made in (a) 13 guinea-pigs inoculated cutaneously at the same time but in different places with live tularemia and brucellosis vaccines; (b) 15 animals vaccinated by the same route with a mixture of these two vaccines and (c) a control group receiving only brucellosis inoculation.

Summarizing the results of these tests, the two authors stated that

- "1. The immunobiological transformation in guinea-pigs inoculated cutaneously and at the same time with tularemia and brucellosis vaccines (separately or in mixture) lead to a weak development of the allergic reactions to tularin and to a considerably more marked reaction to brucellin. The increase of the agglutinin titers in the case of the tularemia antigen and particularly in the case of the brucellosis antigen was considerably less marked than after subcutaneous vaccination. (See Report I.)

2. In the guinea-pigs inoculated cutaneously with brucellosis vaccine alone, the increase in the titer of agglutinins against the brucellosis antigen was slight, but an allergic reaction to brucellin was noted in a majority of the animals.

3. Notwithstanding the presence of low agglutination titers and weak tularin reactions the guinea-pigs cutaneously inoculated with tularemia and brucellosis vaccines proved resistant to challenge with massive doses of a virulent tularemia strain.

4. The guinea-pigs cutaneously inoculated with brucellosis vaccine as well as the animals combined vaccinations were resistant to infection with a virulent Br. melitensis strain."

Pilipenko and his associates (1956), evaluating the efficacy of combined vaccination against tularemia and brucellosis by the intracutaneous route, found that

"1. Simultaneous administration of tularemia and brucellosis vaccines (in mixture or separately) to guinea-pigs by the intracutaneous method produced in the animals an immunobiological transformation in respect to the causative organisms of both infectious (positive allergic reactions to tularin and brucellin and positive agglutination reactions with tularemia and brucellosis antigens).

2. Most suitable (i. e. effective and tolerable) proved a vaccine mixture containing in 0.1 ml one million of tularemia bacilli and 100 million of brucellae. The intracutaneous administration of such doses caused in the guinea-pigs a moderate local inflammatory reaction and produced an intense immunity against the causative organisms of tularemia and brucellosis.

3. An increase of the dosages in the vaccine mixture to 200 million brucellae and 4 million tularemia bacilli led to a severe local reaction with necrosis. A brucella vaccine with a standard lowered to 10 or 1 million, regardless whether it was mixed with tularemia vaccine (with a standard of 10,000 organisms) or used alone, did not produce an immunity against 2-3 infective doses of Br. melitensis. The lowering of the dose of the tularemia vaccine did not reduce the formation of an immunity against tularemia in a noticeable manner."

In an article published in July 1957, in the introduction to which she drew attention to the early work of Pilipenko and Poliakova, Gubina stated that

"In 1953 we commenced studies on the vaccinal process in regard to brucellosis infection after combined immunization against brucellosis and tularemia and also on the intensity and duration of the immunity following various combined vaccinations."

Gubina then proceeded to record observations on 42 guinea-pigs, one group of which had been subcutaneously injected with a mixture of a 1 billion dose of the live Br. abortus BA vaccine and a dose of 10 million organisms of a live tularemia vaccine

Tularemia-II/66

prepared from Gaiskii's strain 15. The second group of animals was immunized only with the latter dose. The test animals were gradually sacrificed for the purpose of bacteriological and histological examinations at intervals ranging from 3 hours to 6 months, but hand in hand with these studies occasion was taken for the performance of serological and allergic tests. As far as tularemia was concerned, it was found that (a) the agglutinin titer began to rise 3 days after administration of either the combined vaccine or tularemia monovaccine, reached a maximum after 20-30 days and then dropped rather rapidly; and (b) tularin tests (like those with brucellin), made 25 days after vaccination, gave positive results with only two exceptions.

Summarizing the results of the histological examinations, Gubina drew attention to a considerable hyperplasia of the reticulo-endothelial system and the early formation of epitheloid nodules in the regional lymph nodes, adding that

"These manifestations were evidently due to the administration of the tularemia vaccine, since brucellosis vaccine alone did not cause an early formation of epitheloid nodules."

In a subsequent paper Gubina (1957 b) referred to observations on 160 guinea-pigs divided into three groups, one of which was immunized cutaneously with a mixture of (a) a live brucellosis vaccine (strains Br. abortus BA or M) with a bacterial standard of 5 billion per ml and (b) NIEG tularemia vaccine containing 100 million organisms per ml; the second with a combined vaccine, in which the standard of tularemia bacilli had been lowered to 1 million; and the third with brucellosis vaccine alone. The conclusion reached by Gubina through these investigations was that the cutaneous administration of the combined vaccine produced a somewhat less intense and shorter lasting immunity than inoculation with brucellosis (or tularemia) monovaccines.

However, fully satisfactory results of combined immunization against tularemia and brucellosis by the cutaneous route were recorded by Shlygina (1958). For her experiments she used mainly ex tempore prepared mixtures of brucellosis vaccines prepared from the Br. abortus BA or M strains and the NIEG tularemia vaccine. One group of 47 guinea-pigs was inoculated with such a combined vaccine containing 50 billion brucellae and 1 billion tularemia bacilli while a second group of 47 animals was immunized with an analogously mixed

Tularemia-II/67

vaccine in which the bacterial standard of the tularemia bacilli had been lowered to 10 million per ml. Two control groups were inoculated with tularemia monovaccines containing respectively 1 billion and 10 million organisms per ml.

Important supplementary tests on 12 guinea-pigs were made with a dry combined vaccine, for the preparation of which use was made of

"Gaiskii's reconstituted tularemia strain No. 15 and (the strain) Br. abortus BA. The mixed vaccines were dried. When determining the amount of live organisms in the vaccine after drying it was found that, after addition of 1 ml of the fluid for suspension, each ampule contained 1 billion live tularemia bacilli and 22.5 billion brucellae."

Summarizing the results of the tests made with the above described vaccines, Shlygina stated that

- "1. Cutaneous administration of a combined brucellosis-tularemia vaccine to guinea-pigs led to the appearance of a stable immunity against tularemia. As shown by tests made after one month, the immunity was as intense as that in the animals inoculated with tularemia monovaccine; 5 months after immunization it was somewhat lower.
2. Immunization with the tularemia vaccine in association with the brucellosis vaccine did not impede the production of antibodies and the appearance of an allergic state in the guinea-pigs due to the tularemia antigens (observations after 1-5 months).
3. The combined vaccine, containing 1 billion tularemia bacilli and 50 billion of brucellae per ml, as well as tularemia monovaccine of a corresponding standard, if cutaneously administered, led within 1-3 months to a higher production of antibodies and also to a somewhat higher survival rate in animals challenged 5 months after immunization than the combined vaccine containing only 10 million tularemia bacilli per ml.

Tularemia-II/68

4. The dry live combined brucellosis-tularemia vaccine (trial series) proved highly efficacious in tests on guinea-pigs, so that it became permissible to use it in man."

The following records on the combined use of tularemia and brucellosis vaccines in man are available:

- (a) This method seems first to have been used by Amanzhulov and Rementsova (1958)¹ who administered to 172 persons with negative tularin and brucellin reactions subcutaneous injections of live brucellosis vaccine into one arm and cutaneous inoculations of live tularemia on the other arm.

Finding that the subcutaneous administration of the brucellosis vaccine was apt to cause frequent and marked reactions, the two workers resorted to cutaneous inoculation of this as well as of the tularemia vaccine in the case of a second group of 200 persons. Summarizing their results, the authors stated that

1. Simultaneous subcutaneous administration of dry live brucellosis vaccine and cutaneous inoculation of dry live tularemia vaccine are harmless and produce inconsiderable reactions. These could be mitigated through cutaneous administration of both vaccines.

2. In the persons simultaneously vaccinated by the cutaneous route with tularemia vaccine and subcutaneously with brucellosis vaccine an immunological transformation took place which could be detected 30 days after the immunization with the aid of Wright's reaction in 91% with Huddleson's reaction in 95% and with Burnet's reaction in 81%, and through tularin tests in 81%. Five months after immunization the number of persons showing positive serological and allergic reactions in respect to brucellosis and tularemia was increased to 97-100%. One year after immunization these reactions were still positive in not less than 56.7%.

3. In the simultaneously vaccinated persons the subcutaneous administration of brucellosis vaccine caused a general reaction in 7% and a local reaction, disappearing

1. Papers on the same subject published by Amanzhulov in 1956 and 1959 and also an article in point by Gudoshnik and Egorova (1959) could not be consulted.

Tularemia-II/69

after 2-3 days, in 100%. At the site of cutaneous tularemia inoculation vesicles began to appear 3-4 days after vaccination in 24% and from the 5th to the 20th day in 76% of the immunized persons.

4. Cutaneous vaccination against tularemia combined with the subcutaneous administration of brucellosis vaccine led to an enlargement of the regional lymph nodes in 86%; if both vaccines were administered cutaneously, such an enlargement of the lymph nodes was noted in only 8% of the vaccinated persons.

5. Simultaneous vaccination against tularemia and brucellosis in the enzootic areas renders it easier to organize measures against these infections.

- (b) Gubina and Uglovoi (1958), making tentative use of combined immunization against tularemia and brucellosis in 1956, obtained such encouraging results that they proceeded to apply this method to a group of 185 persons reacting negatively in preliminary tests with tularin and brucellin. For this purpose they utilized a dry vaccine manufactured with the strains Br. abortus BA and Gaiskii's tularemia strain 15, which after suspension contained per ampule 22 billion of brucellae and 1.5 billion of tularemia bacilli. The contents of one ampule were used for the inoculation of 15 persons with 2 drops of the combined vaccine each.

Results of (a) cutaneous administration of the combined vaccine and (b) inoculation with the corresponding monovaccines of smaller groups of persons who had reacted positively to tularin or brucellin, are shown in the following table:

<u>Kind of Vaccine</u>	<u>Number Inoculated</u>	<u>Allergic State Before Immunization</u>	<u>Number of Takes</u>	<u>Reaction</u>	
				<u>Enlargement of Lymph Nodes</u>	<u>Weakness and Headache</u>
Combined	185	Normal	183	11	2
Brucellosis	17	Tularin-positive	17	1	1
Tularemia	31	Brucellin-positive	30	2	1

Use of the combined vaccine was made also in two groups of 14 persons each who had been found to react positively in

Tularemia-II/70

preliminary tests with brucellin or tularin. After the vaccination among the brucellin-positive persons fever up to 39°C was noted in 2 instances and a marked swelling of the regional lymph nodes in three. In the 14 persons with a positive tularin reaction immunization was followed by fever up to 38.2°C in one, by an usually inconsiderable enlargement of the lymph nodes in 6 instances. Quoting the history of a brucellin-positive individual in whom a marked post-vaccinal reaction had been noted, the author maintained that

"In persons positively reacting to brucellin, the administration of the combined vaccine may possibly lead to stormy general reactions. Therefore in vaccination campaigns with the combined vaccine it is necessary to make preliminary tests with brucellin. Tularin tests are not obligatory."

- (c) Large-scale use of combined vaccination against tularemia and brucellosis was made by Bakker (1957) who in the introduction to the note describing this work stated that

"As a prophylactic measure against brucellosis and tularemia in the civilian population engaged in the Pavlodar Oblast with the grain harvest a vaccination campaign was conducted simultaneously with the dry brucellosis vaccine produced by the Gamaleia IEM (Institute of Epidemiology and Microbiology) for subcutaneous administration and the dry tularemia vaccine manufactured by this institute for cutaneous inoculation. For the sake of convenience both vaccines were administered by the cutaneous route. No preliminary serological examinations (reactions of Wright, Huddleson and Burnet) and no tularin tests were made. A total of 42,300 persons was vaccinated in the oblast."

Inoculation against brucellosis were administered on the right arm in a dose of 400 million organisms (fifth part of an ampule), those against tularemia in the usual dose on the left arm.

Tests made two months after this combined vaccination in a group of 259 persons gave the following result: Wright's reaction was positive in 68 persons (26.25%), usually at titers of 1:100-1:200, thrice only at 1:800; Huddleson's test gave a positive result in 166 persons (64.09%) at titers from 1:100-1:400; Burnet's allergic reaction proved positive in only 25 persons (9.65%). Positive results of the tularin test were obtained in 167 instances (64.48%).

Tularemia-II/71

Observations in an additional group of people who had been cutaneously inoculated in two places of one arm with one drop each of the ex tempore mixed tularemia and brucellosis vaccine showed that

"The local reaction after immunization (appearing) in some persons was slight;...a general reaction (consisting of an enlargement of the cervical and axillary lymph nodes) was observed rarely-- in 0.91%, and reactions in the form of fever or weakness were absent.

...An immunological transformation in respect to the brucellosis was observed in 95.1% of the vaccinated persons, and in respect to tularemia in 86.3%."

No instances of human brucellosis were observed during the harvest, even though many of the participants live under insanitary conditions and consumed raw water and unboiled milk.

- (3) Tularemia and plague. - Since, as will be discussed below, the administration of plague vaccine together with that of vaccines against tularemia and brucellosis or in combination with still other prophylactics has been studied by several workers, it is necessary first to deal with the investigations of Kalacheva (1958, 1959, 1960) on simultaneous immunization against tularemia and plague alone.

In her first paper Kalacheva recorded the results of the combined vaccination of guinea-pigs with live plague and tularemia vaccines in the form of four instructive tables which are quoted below in a somewhat modified form:

(a) Combined Subcutaneous Vaccination Against Plague and Tularemia

<u>Group</u>	<u>Number Tested</u>	<u>Type of Vaccine</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		
					<u>Dose (DCL)</u>	<u>Number Tested</u>	<u>Survived</u>
I	40	Combined (EV strain and ? Gaiskii's strain 15)	1-1.5 bil-lion <u>P. pestis</u> + <u>B.tularensis</u>	<u>P. pestis</u>	100	20	6
				<u>B.tular.</u>	1,000	20	20
II	15	EV strain	1-1.5 bil-lion	<u>P.pestis</u>	100	15	11

Tularemia-II/72

<u>Group</u>	<u>Number Tested</u>	<u>Type of Vaccine</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		<u>Sur- vived</u>
					<u>Dose (DCL)</u>	<u>Number Tested</u>	
III	15	Tularemia	200,000 organisms	<u>B.tular.</u>	1,000	13	13
VI	14	None (controls)	-	<u>P.pestis</u>	100	7	0
				<u>B.tular.</u>	1,000	7	0

The combinedly vaccinated guinea-pigs of this series were thus insufficiently protected against challenge with a virulent plague strain one month after immunization. Better results were obtained in this respect in a second lot of animals subcutaneously vaccinated with the same doses of the EV vaccine but with lesser doses of tularemia bacilli:

<u>Group of Animals</u>	<u>Number Tested</u>	<u>Type of Vaccine</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		<u>Sur- vived</u>
					<u>Dose (DCL)</u>	<u>Number Tested</u>	
I	30	Combined	1 billion	<u>P.pestis</u>	100	15	13
			<u>P. pestis</u> - 10,000 - 100,000 <u>B.</u> tularense	<u>B.tular.</u>	1,000	15	15
II	15	Plague (EV)	1 billion	<u>P.pestis</u>	100	15	13
III	15	Tularemia	10,000 - 100,000 organisms	<u>B.tular.</u>	1,000	15	15

N.B. The control animals challenged with plague and tularemia bacilli respectively all succumbed.

Since even in this series of tests the results obtained with the EV strain were not fully satisfactory, a further group of animals was subcutaneously immunized with the vaccinal plague strains 1 and 17 in combination with Gaiskii's tularemia strain No. 15. The results are shown in the following table:

Tularemia-II/73

<u>Group of Animals</u>	<u>Number Tested</u>	<u>Type of Vaccine</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		<u>Survived</u>
					<u>Dose (DCL)</u>	<u>Number Tested</u>	
I	25	Fluid combined	500 million each of plague strains 1 and 17 +	<u>P.pestis</u>	100	15	14
			10,000 orgs. of Gaiskii's strain No.15	<u>B.tular.</u>	100	10	10
II	10	Fluid plague di-vaccine (strains 1 and 17)	500 million organisms of each strain	<u>P.pestis</u>	100	10	10
III	8	Fluid plague vaccine (Strain 1)	500 million organisms	<u>P.pestis</u>	100	8	7
IV	8	Dto. (Strain 17)	-"-	-"-	100	8	5
V	8	Fluid tular-emia vaccine	10,000 organisms	<u>B.tular.</u>	1,000	8	8

N. B. The control animals challenged with plague and tularemia bacilli respectively all succumbed.

It will be seen that the combined vaccine made with the plague strains 1 and 17 gave slightly better results than that for the manufacture of which the EV strain had been used (93% as against 86% survivals after challenge).

(b) Combined Cutaneous Vaccination Against Plague and Tularemia

The results of combined cutaneous vaccination against plague and tularemia (strains EV and Gaiskii No. 15), as established through challenge tests one month after immunization, are shown below:

Tularemia-II/74

<u>Group of Animals</u>	<u>Number Tested</u>	<u>Type of Vaccine</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		
					<u>Dose (DCL)</u>	<u>Number Tested</u>	<u>Survived</u>
I	10	Dry combined (containing after suspension tularemia bacilli per ml)	2 drops	<u>P.pestis</u>	100	5	5
				<u>B.tular.</u>	1,000	5	5
II	5	Dry plague (standard as above)	2 drops	<u>P.pestis</u>	100	5	5
III	4	Dry tularemia (standard as above)	2 drops	<u>P.tular.</u>	1,000	4	3

N. B. The control animals challenged with plague and tularemia bacilli respectively all succumbed.

Challenge tests with virulent plague or tularemia cultures in guinea-pigs which had been immunized with the above mentioned vaccines 6 months previously, gave the results shown in the following tabulation:

<u>Group of Animals</u>	<u>Number Tested</u>	<u>Type of Vaccine and Mode of Vaccination</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge</u>		
					<u>Dose (DCL)</u>	<u>Number Tested</u>	<u>Survived</u>
I	20	Dry combined - Subcutaneous	1 billion plague + 100,000 tularemia bacilli	<u>P.pestis</u>	100	10	5
				<u>B.tular.</u>	1,000	10	8
II	10	Plague - Subcutaneous	1 billion organisms	<u>P.pestis</u>	100	10	6
III	10	Tularemia - Subcutaneous	100,000	<u>B.tular.</u>	1,000	10	8

(Table continued on page 75)

Tularemia-II/75

<u>Group of Animals</u>	<u>Number Tested</u>	<u>Type of Vaccine and Mode of Vaccination</u>	<u>Vaccine Dose</u>	<u>Strain</u>	<u>Challenge Dose (DCL)</u>	<u>Number Tested</u>	<u>Survived</u>
I	30	Dry combined (standard after 100 billion plague and 2 billion tularemia bacilli per ml) - Cutaneous	2 drops	<u>P.pestis</u>	100	14	11
				<u>B.tular.</u>	1,000	14	14
II	15	Plague (standard as above) - Cutaneous	"-	<u>P.pestis</u>	100	15	11
III	15	Tularemia (standard as above) - Cutaneous	"-	<u>B.tular.</u>	1,000	15	12

N. B. The control animals challenged with plague and tularemia bacilli respectively all succumbed.

Thus 50% of the animals immunized subcutaneously with the combined vaccine were after 6 months still resistant to plague infection, 80% to tularemia infection. The corresponding figures after cutaneous inoculation were 78% and 100%. It will be seen, therefore, that in these as in most of the other tests immunization against tularemia gave better results than that against plague.

Studying the leucocytic reactions in white mice immunized respectively with a live combined vaccine against tularemia and plague and with the corresponding monovaccines as well as in not vaccinated control animals Kalacheva (1959) found that in the latter four hours after intraperitoneal infection with plague bacilli the peritoneal exudate contained a large amount of the organisms and only few leucocytes (2-5 per field of vision). Blood cultures taken from these animals usually showed an abundant growth of P. pestis. In the peritoneal smears from intraperitoneally infected plague-vaccinated animals only single (or sometimes even no) plague bacilli were seen and at an average 10 leucocytes were found per field of vision. Blood cultures gave the same results as in the controls.

In smears from the peritoneal exudate of mice immunized with the combined vaccine and challenged with plague, P. pestis

Tularemia-II/76

were rare or absent but there was a marked leucocytosis (15-20 cells per field of vision). Blood cultures from these animals yielded only single colonies or were even negative. In control mice infected with tularemia there was a slight leucocytic reaction (1-5 cells per field of vision). Blood cultures showed an abundant growth of tularemia bacilli.

In mice immunized with tularemia vaccine and challenged intraperitoneally with B. tularensis there was a marked leucocytosis in the peritoneal exudate (10 or more cells per field of vision). When the blood of animals killed 4 hours after challenge was cultivated on egg-yolk media, positive results were obtained in only 5 out of 20 instances.

In mice immunized with the combined vaccine and challenged with tularemia, there was a marked leucocytic reaction (more than 20 cells per field of vision). Blood cultures were sterile in 75% or less abundant than in the controls.

Thus, Kalacheva maintained, the combined vaccine conferred a higher degree of protection than the monovaccines. There was no competition among the antigens - on the contrary sometimes an intensification of the protective reaction.

In the opinion of this worker, the peritoneal test was more valuable for an assessment of the state of immunity after vaccination - it was almost uninfluenced by the intoxication of the animals and revealed the anti-infectious character of the immunity.

In her third paper (1960), Kalacheva reported on the simultaneous cutaneous administration of the dry plague vaccine manufactured in the "Mikrob" Institute (Saratov) and the dry tularemia vaccine of the Gamaleia Institute to a group of 60 persons. Evidently she used two separate sets of scarifications for inoculation with each of these monovaccines and a third for the administration of a mixture of both. She found that in these 60 persons

"the local reaction to the plague vaccine was positive in 57, being marked in 34 instances and slight in 23 persons. It appeared at the end of the first day and lasted for 3-4 days. In the case of a marked reaction one noted a bright reddening of the skin at the site of inoculation, swelling and the presence of vesicles along the scarifications. The vesicles were absent in the case of slight reactions.

Tularemia-II/77

The local reaction to the tularemia vaccine appeared as a rule between the 7th and 10th day, but rarely earlier or later. It was marked in 50 of the inoculated persons and slight in 7."

A general reaction was noted in 13 out of these 60 persons, consisting of a slight rise of the body temperature in 5 instances, higher fever once, headache in 6 persons and enlargement of a regional lymph node in one instance.

The local and general reactions following the administration of plague and tularemia monovaccines in two control groups were as follows:

<u>Monovaccine Used</u>	<u>L o c a l R e a c t i o n s</u>			<u>G e n e r a l R e a c t i o n s</u>		
	<u>Marked</u>	<u>Slight</u>	<u>Absent</u>	<u>Marked</u>	<u>Slight</u>	<u>Absent</u>
Plague	7	10	3	-	3	17
Tularemia	10	8	2	1	3	16

N. B. The general reactions consisted of headache, associated once with the enlargement of a regional lymph node.

Tularin tests made 3 weeks after the simultaneous administration of the two vaccines gave a markedly positive result in 37 instances, a slightly positive result in 20 persons.

Kalacheva concluded from these observations that the simultaneous use of plague and tularemia vaccines was justified.

- (4) Tularemia, brucellosis and plague. - The problem of combined immunization against tularemia, brucellosis and plague has been the subject of studies by Klets and his associates (1958 a, b, 1959 a, b), Pilipenko and co-workers (1959, 1960, 1961, 1963 a, b) and Uzbekova (1962).¹ To which of these observers priority

1. The 1958 conference report by Klets and co-workers as well as that rendered by Pilipenko in 1959 could not be consulted. The same holds true of the monograph Brutsellez i tuliaremiia (Brucellosis and Tularemia) published by Pilipenko et al. at Stavropol' in 1959 and of their contribution to the book Assotsiirovannaia vaktsinatsiia (Combined vaccination), Moscow, 1959.

Tularemia-II/78

belongs, is difficult to decide. Klets (1959 a) declared to "have met with no publications dealing with simultaneous immunizations with live combined vaccines against plague, tularemia and brucellosis." However, as will be discussed below, Pilipenko and his associates (1960) referred to earlier investigations of this problem and stated that they themselves had commenced to study it in 1955. Nevertheless, for the convenience of record it is proposed to begin the present disquisition with a review of the observations of Klets and his co-workers.

In their short note published in the Zh. mikrobiologii... (1958 b) as well as in the report embodied in the 20th volume of the records of the Irkutsk Anti-Plague Institute (1959 a) these observers referred to tests in guinea-pigs subcutaneously immunized with (a) a combined vaccine ex tempore prepared by mixing suspensions from two days old cultures of Gaiskii's tularemia strain 15, the Br. abortus strain BA and the vaccinal plague strains 1 and 17, used in dosages of respectively 25 million organisms, 250 million organisms and 1.5 billion organisms (750 million of each of the two plague strains); and (b) the corresponding monovaccines. Challenge tests made 36 days after immunization gave the following results:

<u>Kind of Vaccine Used</u>	<u>Results of Challenge Tests with Virulent Cultures of</u>					
	<u>B. tularensis</u>		<u>Brucella ovis</u>		<u>P. pestis</u>	
	<u>Tested</u>	<u>Survived</u>	<u>Tested</u>	<u>Proved Immune</u>	<u>Tested</u>	<u>Survived</u>
Combined (tularemia, brucellosis and plague)	14	14	14	11	14	12
Monovaccines against tularemia, brucellosis and plague	15	14 ^a	15	13	11	6 ^b
None (controls)	9	1 ^c	9	0	10	0
<u>Remarks.</u> - (a) The 15th animals succumbed to an unspecific infection. (b) 4 of the five succumbing animals showed signs of an unspecific infection. (c) Challenged with a minimal dose.						

On account of these observations the authors considered it permissible to use the three vaccines studied by them in combination.

Tularemia-II/79

In the introduction to their second 1959 report Klets and his associates maintained that their previous observations (1956-1959) had proved the efficacy of a combined immunization against the three above mentioned infections. However, in order to demonstrate the practical usefulness of this method, they had found it necessary to make further tests with mixtures of the dry live vaccines available for the prophylaxis of these diseases. The combined vaccine obtained by mixing saline suspensions of these vaccines contained per ml (the dose used) 25 million tularemia bacilli, 250 million brucellae and 750 million each of the two vaccinal plague strains (1 and 17). Challenge tests made 6 months after immunization in guinea-pigs subcutaneously injected with this combined vaccine, which was well tolerated by the animals, and control tests with the corresponding monovaccines gave the results shown below:

<u>Kind of Vaccine Used</u>	<u>Results of Challenge Tests with Virulent Cultures of</u>					
	<u>B. tularensis</u>		<u>Brucella ovis</u>		<u>P. pestis</u>	
	<u>Tested</u>	<u>Survived</u>	<u>Tested</u>	<u>Proved Immune</u>	<u>Tested</u>	<u>Survived</u>
Combined	20	18*	18	8	20	9
Monovaccines against, brucel- losis and plague	19	19	19	8	20	8
None (normal controls)	10	0	10	0	10	0

* The two remaining animals succumbed to unspecific infections.

Klets and his associates concluded from these findings and also from tests with combined vaccines with an up to five times decreased bacterial concentration

"that the live dry combined vaccine against plague, tularemia and brucellosis, composed of fully compatible strains, when tests on guinea-pigs causes slight reactions and possesses an exactly marked immunizatory efficacy - which permits the use of this vaccine for combined vaccination against plague, tularemia and brucellosis."

An unbiased observer cannot help but note, however, that the combined vaccine proved fully satisfactory only with regard to protection against tularemia.

Tularemia-II/80

In the introduction to their 1960 article Pilipenko and his co-workers, after referring to earlier publications on the simultaneous use of tularemia and brucellosis vaccines, stated without furnishing references that

"A study of the compatibility of plague, tularemia and brucellosis vaccines under the condition of simultaneous inoculation by various workers gave an analogous result: injection of a mixture of these vaccines produced in guinea-pigs an immunobiological transformation in regard to the causative organisms of these infections which was of the same degree as that produced by the mono-vaccines. These results were obtained through subcutaneous vaccination which can hardly find a large-scale practical use. First of all this will be hindered by the marked local post-vaccinal reactions. In the animals subcutaneously injected with a mixture of the three vaccines this reaction usually appeared in the form of subcutaneous infiltrates which sometimes reached a considerable size and firmness. These reactions persisted for 12-18 and sometimes for 24-30 days. In part of the guinea-pigs 2-3 weeks after vaccination one could note in the center of the infiltrate purulent abscesses which afterwards opened."

Summarizing the results of challenge tests in guinea-pigs cutaneously inoculated with the combined vaccine and, for the purposes of control, with the three corresponding mono-vaccines, Pilipenko and his colleagues stated the following:

"While invariably causing local post-vaccinal reactions with a mild course, the efficacy of the method of cutaneous administration of the combined vaccine was not lower than that which had been noted after subcutaneous injection of this vaccine. Two months after vaccination there existed no substantial differences in the number of immune animals between the group inoculated with the combined vaccine and that immunized with the corresponding monovaccines. The brucellosis vaccine showed a somewhat higher efficacy when admixed to the two other vaccines."

The authors admitted, however, that

"6 months after immunization one could note a diminution of the number of animals resistant to massive challenge doses among the animals vaccinated with either the monovaccines or the combined preparation. In this the number of guinea-pigs which had lost the immunity against large challenge doses of plague or tularemia bacilli was about two times higher among the animals inoculated with the combined vaccine than among those inoculated with the corresponding monovaccines."

Commenting upon the observations made 6 months after immunization, Pilipenko and his associates stated that

"Klets et al. (1958), testing the immunity of guinea-pigs 7 months after subcutaneous vaccination with a mixture of plague, tularemia and brucellosis vaccines, found no substantial difference in the animals immune to plague after administration of either the combined or the monovaccine; this difference was slight in the case of tularemia whereas a large part of the guinea-pigs was found susceptible to brucellosis after immunization with either the combined or the monovaccine."

In the opinion of Pilipenko and his colleagues it was still an open question whether the differences in point detected by them were of an intrinsic character or merely accidental and they maintained that these discrepancies did not diminish the superior value of the cutaneous administration of mixtures of the three vaccines.

In the following of their series of studies (1961) Pilipenko and his group reported not only upon the outcome of further challenge tests but also upon observations in guinea-pigs cutaneously inoculated with the combined vaccine in doses of 3 billion plague bacilli (strains 1 and 17), 1.5 billion brucellae (strain 19 BA) and 75 million tularemia bacilli and sacrificed afterwards at intervals ranging from 3 hours to 90 days (or, in the case of tularemia, 60 days). In the former respect it was found that

- (a) In the case of challenge with P. pestis 7 months after inoculation the resistance of the animals immunized with the combined vaccine was two times lower than that in the group vaccinated against plague only;

Tularemia-II/82

- (b) Whereas 5 1/2 months after vaccination only 50% of the guinea-pigs inoculated with the combined vaccine proved immune to brucellosis infection, the corresponding figure in the animals protected through administration of the monovaccine was almost 90%;
- (c) In contrast to the previous findings, the number of combinedly inoculated animals resistant to challenge with 5,000 DCL of a virulent tularemia strain was two times higher than that among the animals protected with the tularemia monovaccine.

The authors added, however, that since all 7 animals which succumbed to tularemia even though they had been inoculated with this monovaccine, had shown signs of allergy and had agglutinins in their sera, the cause of their death "might not have been related to the immunization."

Commenting upon the observations made in the sacrificed animals, Pilipenko and his associates summarized

"that in the guinea-pigs inoculated with the combined vaccine the plague, brucellosis and tularemia bacteria were observed in the body of the test animals less longer than was the case after administration of the monovaccines. Consequently one observed in the former group of animals more rarely a generalization of the vaccinal process."

The shorter duration of the immunity in the animals vaccinated with combinations evidently was causally related to the briefer persistence of the vaccinal organisms in the body of the test animals. Presumably this briefer persistence was explained by the reduced numbers of the organisms of each vaccinal strain which could enter the skin scarifications during the process of combined vaccination. In the opinion of Pilipenko and his co-workers this drawback of the combined method of vaccination might be overcome by an increase of the vaccine doses, changes in the proportion of the components of the combined vaccine or in the technique of cutaneous inoculation.

Again dealing with the problem of combined vaccination against tularemia, brucellosis and plague in an article published in 1963, Pilipenko and his associates pointed out

"that the doses of antigens in the cutaneously administered monovaccines, which ensure the

Tularemia-II/83

production of a stable immunity if administered once, may prove insufficient to produce such an immunity against all three causative organisms in question if given in a combined vaccine."

This seemed to hold true particularly of the tularemia component, since in the combined vaccine the ratio of the tularemia bacilli to P. pestis and the brucellae was only one to 40-60. For this reaction the authors, when making further experiments, used not only a combined vaccine of the usual composition but also one with higher contents of tularemia bacilli (and also of brucellae), as shown in the following tabulation:

<u>Strains Used</u>	<u>Bacterial Standard per Dose (2 drops or 0.1 ml)</u>	
	<u>Series I</u>	<u>Series II</u>
<u>P. pestis</u> (strains 1 and 17 or EV)	3 billion	3 billion
<u>Br. abortus</u> 104 M	2 billion	<u>4 billion</u>
<u>B. tularens</u> (Gaiskij's strain 15 reconstituted)	50 million	<u>250 million*</u>

* The authors were careful to point out that, since opacity tests adjusted to the bacteria of the gastrointestinal group had been used for the standardization of the vaccines, the actual dosage of tularemia bacilli was 1-1.2 billion, i.e. 5-6 times more than the dose generally used for human vaccination (160-200 million organisms).

The results of challenge tests in groups of guinea-pigs immunized respectively with these two sorts of vaccine are shown in the following tables:

(a) Challenge Tests with 200 DCL of a Virulent Plague Strain

<u>Series of Vaccines</u>	<u>Kind of Vaccines</u>	<u>Time After Immunization</u>	<u>Number of Animals Tested</u>	<u>Suc-</u>	<u>Survived</u>	
				<u>cumbed to Plague</u>	<u>Number</u>	<u>Percent</u>
I	Combined (strains 1 and 17)	40 days	23	1	22	95.5
	Plague monovaccine (strains 1 and 17)	"-	12	0	12	100.0

(Table continued on page 84)

Tularemia-II/84

<u>Series of Vaccines</u>	<u>Kind of Vaccines</u>	<u>Time After Immunization</u>	<u>Number of Animals Tested</u>	<u>Succumbed to Plague</u>	<u>Survived Number</u>	<u>Percent</u>
	Combined(as above)	6 months	11	0	11	100.0
	Plague monovaccine (as above)	"-	12	0	12	100.0
II	Combined(EV strain)	50 days	12	0	12	100.0
	Plague monovaccine (EV strain)	"-	12	0	12	100.0

N. B. All controls succumbed to plague.

It will be seen that tests with all the vaccines used gave satisfactory results.

(b) Challenge Tests with 2 Doses of a Virulent Br. melitensis Strain Apt to Cause a Generalized Infection

<u>Series of Vaccines</u>	<u>Kind of Vaccines</u>	<u>Time After Immunization</u>	<u>Number of Animals Tested</u>	<u>Found Immune Number</u>	<u>Percent</u>
I	Combined(plague strains 1 and 17)	43 days	22	20	91.0
	Brucellosis mono-vaccine	"-	12	12	100.0
II	Combined(EV plague strain)	55 days	12	12	100.0
	Brucellosis mono-vaccine	"-	12	12	100.0
	Combined(plague strains 1 and 17)	5 1/2 months	11	9	82.0
	Brucellosis mono-vaccine	"-	12	12	100.0

N. B. None of the controls proved immune.

Tularemia-II/85

It is interesting to note that 5 1/2 months after immunization the combined vaccine of Series II, even though it contained an increased dosage of brucellae, did not afford as complete a degree of protection as the corresponding brucellosis monovaccine.

(c) Challenge Tests with 1,000 DCL of a Virulent Tularemia Strain

<u>Series of Vaccines</u>	<u>Kind of Vaccines</u>	<u>Time After Immunization</u>	<u>Number of Animals Tested</u>	<u>Succumbed to Tularemia</u>	<u>Survived Number</u>	<u>Percent</u>
I	Combined(plague strains 1 and 17)	38 days	22	15	7	31.8
	Tularemia mono-vaccine	"-	12	2	10	83.3
	Combined(as above)	6 months	11	5	6	54.5
	Tularemia mono-vaccine	"-	12	6	6	50.0
II	Combined(EV plague strain)	48 days	12	1	11	91.6
	Plague(EV), brucellosis and tularemia vaccines administered simultaneously but separately	"-	12	0	12	100.0
	Tularemia mono-vaccine	"-	12	0	12	100.0

N. B. In both series of tests all controls succumbed to tularemia.

Thus the vaccines of Series II, which contained higher dosages of tularemia bacilli, gave better results than those of Series I. It has to be noted, however, that challenge tests with the former vaccines (Series II) were made merely 48 days after immunization but not at a later date.

On account of these experiences Pilipenko and his colleagues recommended that for the preparation of combined vaccines a 3-5 times increased dosage of tularemia bacilli be used.

Tularemia-II/86

In a second paper published in 1963 Pilipenko and his colleagues recorded the observations they could make when administering their trivaccine to 54 human subjects. For the purposes of control they inoculated at the same time 21 persons against plague, 33 against brucellosis and 22 against tularemia. The conclusions which could be drawn were that

"1. The cutaneous inoculation of a combined vaccine against plague, tularemia and brucellosis did not lead to severe local or general reactions in the immunized persons. The degree of the manifestation of the local and general post-vaccinal reactions was identical in those inoculated with the combined or the monovaccines.

2. The immunological response (tularin and brucellin reactions, agglutination reaction) was of an identical character in those inoculated (respectively) with the combined or the monovaccines.

3. The combined trivaccine, containing the usual doses of the plague, brucellosis and tularemia antigens for cutaneous inoculation, and also a vaccine containing a 4-5 times increased dose of the tularemia component,¹ proved to be innocuous and capable of producing an immunological response in respect to the antigens constituting the combined product.

4. Regardless whether or not a dry combined vaccine is available, one may organize simultaneous inoculations against plague, tularemia and brucellosis, using for this purpose a mixture prepared before use from the corresponding three dry monovaccines. From the latter one can prepare, if so needed, not only the combined trivaccine but any desired combination of two of the vaccines.

1. The vaccine in question contained per dose (2 drops or 0.1 ml) 3 billion organisms of the EV strain, 2 billion of the 104 M brucellosis strain and 250 million of tularemia bacilli. It was tested in a group of 8 people.

5. Our experimental investigations on guinea-pigs and experiences in man permit consideration of the problem of the combined vaccine against plague, tularemia and brucellosis for cutaneous administration as basically solved."

Earlier attempts to use the method of combined vaccination against plague, tularemia and brucellosis for the immunization of man were made by Borodko and his co-workers (1959) and by Uzbekova and her assistants (1962).

As quoted by Borodko and Samsonovich (1962), the former group of observers had found that this combined vaccine, if used for cutaneous inoculation, was innocuous and produced in the immunized persons an immunological response analogous to that resulting from the administration of the corresponding monovaccines.

Uzbekova and her associates tested altogether 383 volunteers divided into the following groups:

<u>Group</u>	<u>Kind of Vaccine Used</u>	<u>Number Inoculated</u>	<u>Remarks</u>
I	Combined plague-brucellosis-tularemia vaccine.	270	Of this group (a) 207 persons had shown negative results in preliminary brucellin and tularin tests; (b) 40 had been tularin-positive; (c) 17 had been brucellin-positive and (d) 6 had reacted to both allergens.
II	Combined plague and brucellosis vaccine.	83	80 of these persons had been tularin-positive, 3 both tularin- and brucellin-positive.
III	Plague vaccine.	30	3 of these persons had been tularin-positive, 26 tularin- and brucellin-positive, while 1 had not reacted to either of these allergens.
<u>Total</u>		283	

N. B. For the composition of the combined vaccines were used: (i) a plague vaccine manufactured in the Central-Asia Anti-Plague Institute; (ii) a vaccine produced with the Br. abortus 19 strain in the Kashintsevskaja biofabrika and (iii) the standard tularemia vaccine of the Gamaleia Institute.

Tularemia-II/88

Evaluating their findings, Uzbekova and her associates stated that

1. The combined cutaneous inoculation of live vaccines against plague, brucellosis and tularemia produced local reactions and reactions in the regional lymph nodes in only 8.2% of the vaccinated.

2. In persons who had reacted positively to the intracutaneous administration of tularin or brucellin and more still in individuals who had reacted to both these allergens, post-vaccinal reactions were considerably more frequent (35%, respectively 47% and 66.7%). Even then, however, the reactions were short-lasting and not intense.

3. After vaccination with two antigens (plague and brucellosis) or only against plague reactions were noted in a limited number of persons (19.3%, respectively 10%) and were likewise short-lasting and not stormy.

4. After combined vaccination the larger part of the inoculated showed an accumulation of brucellosis agglutinins in their sera, an accumulation which persisted for not less than 6 months. The percentage of persons with positive serological reactions and the agglutinin titers were higher in those vaccinated only against plague and brucellosis than in those given all three vaccines.

5. Burnet's allergic reaction on the contrary was more frequently observed in the latter group than in those vaccinated only against plague and brucellosis.

6. Agglutination tests with tularemia antigen were positive in 73.8% of the persons simultaneously inoculated with all three vaccines. However, the percentage of positive reactions as well as the agglutinin titers were lower than was usual in persons receiving only the tularemia vaccine.

7. Further large-scale tests are necessary for a final decision of the question whether the method of simultaneous cutaneous inoculation against plague, brucellosis and tularemia could be adopted for mass immunization.

- (5) Tularemia, brucellosis and anthrax. - The problem of a combination of anthrax immunization with that against tularemia and brucellosis was investigated by Anina-Radchenko and associates (1959). As quoted by Pilipenko and Miroshnichenko (1963), these workers,

"studying the possibility of simultaneous vaccination against tularemia, brucellosis and anthrax, came to the conclusion that the low immunological efficacy in regard to brucellosis and tularemia detected by them was due to the presence of the anthrax antigen in the combined vaccine. In their turn the brucellosis and tularemia vaccines, or perhaps one of them, conditioned a lowering of the immunological efficacy of the anthrax vaccine used in combination with these vaccines."

- (6) Tularemia, plague, brucellosis and anthrax. - As stated by Vereninova and her co-workers in an article published in 1958, experimental studies on the possibility of combining immunization against anthrax with that against tularemia, plague and brucellosis were commenced by her group and also independently by Pilipenko and his associates in 1955.

In the first of their valuable contributions to this problem Vereninova and her co-authors (1958) recorded observations in 453 guinea-pigs which were immunized in the following ways:

- (a) The first group was subcutaneously injected with a mixture of all four vaccines under test;
- (b) The second series of animals was given simultaneously a mixture of the tularemia and plague vaccines by the cutaneous route and a mixture of the brucellosis and anthrax vaccines by subcutaneous injection;
- (c) In the third series of experiments the four vaccines, though also given simultaneously, were each applied at a separate site, the plague and tularemia vaccines cutaneously, those against brucellosis and anthrax by the subcutaneous route;
- (d) In the fourth series of tests the brucellosis vaccine was injected subcutaneously while the other three vaccines were administered cutaneously--the tularemia and plague vaccines in mixture at one site, the anthrax vaccine at another place.

Commenting on the findings in the first group of animals, the authors stated that

"as a result of the combined vaccination one observed a high resistance of the guinea-pigs to challenge with plague and tularemia bacilli

Tularemia-II/90

and the absence of generalized brucella infections. About the same survival rate was observed in the animals immunized with the monovaccines and challenged with the appropriate strains."

At the same time one could note a marked inhibition of the anthrax antigen administered together with the three other antigens: while 8 out of the 10 guinea-pigs immunized with anthrax monovaccine resisted challenge with a virulent B. anthracis culture, only one of the 10 animals immunized with the tetravaccine survived an identical challenge.

In explanation of this discrepancy Vereninova and her co-authors postulated that

"The anthrax vaccine was apparently a weaker antigen and consequently it comes under the influence of the complex of the more active antigens (tularemia, plague and brucellosis) to an inhibition of the protective mechanism against anthrax infection."

Identical findings were made in the other three series of tests, as exemplified by the following results of challenge tests with B. anthracis in the animals of the fourth group:

<u>Kind of Vaccination</u>	<u>Challenged with</u>		<u>Mixture of</u>	
	<u>B. anthracis</u>		<u>Virulent Plague, Tularemia and</u>	
	<u>Tested</u>	<u>Survived</u>	<u>Anthrax Bacilli and of Brucellae</u>	
			<u>Tested</u>	<u>Survived</u>
Combined	10	3	11	3
Anthrax monovaccine	10	9	-	-
None(normal controls)	10	0	10	0

N. B. In this as in all other experiments of Vereninova and her associates the animals were challenged 30 days after immunization.

The inhibition of the anthrax component of the combined tetravaccine was also confirmed by the observation

"that in the animals inoculated with anthrax monovaccine which died before challenge (during the vaccination period) cultures of the vaccinal strain could be isolated in almost 100%, whereas

from the animals succumbing during the corresponding period after combined vaccination B. anthracis could be cultivated very rarely, (and then) mainly from the site of infection and the regional lymph nodes."

In an attempt to improve the results of immunization against anthrax, Vereninova and her co-workers resorted to subcutaneous administration of the anthrax vaccine 10 days before or after immunization with the three other vaccines by the same route. Results of this modified procedure were satisfactory, especially if the anthrax vaccine was first administered.

In a second article (1959) Vereninova et al. recorded observations on 300 guinea-pigs which after simultaneous immunization against plague, tularemia, brucellosis and anthrax were challenged 25 days later by the intratracheal route. The conclusions reached through these tests were that

- "1. Under the condition of intratracheal challenge one could detect a sufficiently intense immunity in guinea-pigs immunized with live plague, tularemia and brucellosis vaccines.
2. Under the same conditions of challenge the anthrax antigen exhibited an insufficient activity, as shown by the extraordinarily low survival rate of the animals immunized with anthrax vaccine 10 days before administration of the complex of the three other vaccines, and also of the animals immunized only with anthrax monovaccine.
3. In agreement with the low efficacy of the anthrax vaccine manifested in intratracheal challenge...one could observe a low survival rate in the group of guinea-pigs challenged (after combined vaccination) with a mixture of all four infecting agents.
4. As shown by comparative studies on the efficacy of the plague vaccine in relation to the method of its administration, best results were obtained with the method of cutaneous administration."

It was not possible to consult a third article published by Vereninova and her associates in 1960 which dealt with the duration of the immunity in guinea-pigs simultaneously vaccinated against plague, tularemia, brucellosis and anthrax.

Tularemia-II/92

As quoted by Pilipenko and Miroshnichenko (1963), the early studies of Pilipenko and his co-workers (1959 a, b) led to conclusions analogous to those arrived at by Vereninova and her group. However, divergent views held by other observers led Pilipenko and Miroshnichenko to a further profound study of the problem of combined vaccination against the four infections presently discussed. They experimented for this purpose with two groups of guinea-pigs, namely, (a) such which were immunized simultaneously with all the four vaccines under study through cutaneous administration of a mixture of the EV or 1/17 plague vaccine, the 104-M brucellosis vaccine and tularemia vaccine produced with the (presumably reconstituted) strain No. 15 of Gaiskii on one side of their abdomen and cutaneous inoculation with two drops of the STI anthrax vaccine on the other side of their belly; and (b) animals inoculated with the last mentioned vaccine at various intervals before the combined administration of the other three vaccines.

Results of challenge tests made with 10 DCL of TSenkovskii's vaccinal anthrax strain No. 2 at different intervals after these various methods of immunization had been used, are summarized in the following table:

<u>Series and Kind of Vaccine</u>		<u>Animals</u> <u>Tested</u>	<u>Succumbed</u> <u>to Anthrax</u>	<u>Survived</u>	<u>Percentage</u> <u>of Survival</u>
I	Combined (Plague strains 1/17)	12	6	6	50.0
I -	Combined (Plague strain EV)	12	8	4	33.3
II	Combined (Plague* strains 1/17)	12	1	11	91.3
	STI vaccine only	12	0	12	100.0
	None (controls)	12	12	0	0.0

* The anthrax vaccine was administered 16 days before inoculation with the three other vaccines.

There was thus a striking difference in the survival rate of the animals inoculated simultaneously against all four infections on the one hand and those receiving the anthrax vaccine alone or previously on the other hand.

As emphasized by the authors,

"This difference was paradoxically related to the character of the vaccinal process. In the guinea-pigs inoculated only with the STI strain, the organisms of this strain could be observed in the organs for not longer than 15 days, i.e. they disappeared about two times more rapidly than was the case in the animals vaccinated with combinations; the number of immune animals, on the contrary, was higher among those immunized with the monovaccine. This shows that the other live vaccines did not exert a direct harmful action on the STI vaccine. This is also proved by the simultaneous growth of the organisms of the STI, plague and brucellosis vaccines in cultures from the lymph nodes of the immunized animals. On the contrary, in association with the latter organisms the STI bacilli lived considerably longer in the body of the immune guinea-pigs. A probable explanation is that the presence of the three other vaccines brought about an inhibition not of the anthrax bacillus but of its protective antigen by which, as has been established within recent years, the immunogenicity of B. anthracis is constituted."

The two authors postulated in this connection that

"In the body of guinea-pigs, in which no other live vaccines are present, evidently this antigen does not become rapidly destroyed or weakened and as a result in such cases there develops in all animals a considerable anti-anthrax immunity, which hastens the destruction of the STI organisms in their body. One gets the impression that the STI vaccine, alone introduced in the body of guinea-pigs, 'works against itself' more quickly than in the case of its introduction simultaneously with other live vaccines. The long persistence of the STI vaccine in the body of guinea-pigs vaccinated with combinations is most probably a sign of the incompleteness of the immunity against anthrax in such cases."

As shown by the following tabulation, embodying the results of challenge tests with TSenkovskii's vaccinal anthrax

Tularemia-II/94

strain No.2 3 1/2 months after immunization, a total inhibition of the immunogenic action of the STI vaccine took place when it was administered simultaneously with two of the other vaccines under study in any combination:

<u>Kind of Vaccine</u>	<u>Animals Tested</u>	<u>Succumbed to Anthrax</u>	<u>Survived</u>	<u>Percentage of Survival</u>
Plague, tularemia, brucellosis and STI	12	11	1	8.0
Plague, brucellosis and STI	12	12	0	0.0
Plague, tularemia and STI	12	12	0	0.0
Brucellosis, tularemia and STI	12	12	0	0.0
STI only	12	4	8	66.0
None (controls)	12	12	0	0.0

On the other hand no evidence for a harmful influence of the STI vaccine on the immunogenic activity of the other three vaccines could be found in numerous tests, in which cutaneous administration of the anthrax vaccine at one site was combined with simultaneous inoculation of mixtures of the other three vaccines on another part of the skin of the test animals.

As pointed out by Pilipenko and Miroshnichenko, their findings were in agreement with those of Vereninova and her group, but contradicted the postulation of Anina-Radchenko et al. that the STI vaccine on the one hand, the plague and brucellosis vaccines on the other hand exerted an inhibitory action on each other. The results obtained by Pilipenko and Miroshnichenko were also not in accord with those of Borodko and Samsonovich (1959) who, studying the compatibility of plague, tularemia, brucellosis and anthrax vaccines, found no evidence for an inhibitory action of any of these prophylactics. However, as will be discussed now, further observations induced these two workers to modify the opinion they had formerly held in this respect.

In order to study the influence of re-vaccination on guinea-pigs which had been vaccinated simultaneously

against plague, tularemia, brucellosis and anthrax or against the three first mentioned infections, Borodko and Samsonovich (1961) re-vaccinated groups of the thus immunized animals by the cutaneous route with the corresponding monovaccines or with the combined tri- or tetravaccine. They used for this purpose (a) a plague vaccine produced in the Saratov "Mikrob" Institute; (b) a tularemia vaccine obtained from the Odessa Institute of Epidemiology and Microbiology; (c) a brucellosis vaccine manufactured in the Gamaleia Institute and (d) STI vaccine from the Tbilisi Vaccine and Serum Institute.

Tests with tularin and brucellin made one month after re-vaccination revealed no change in the allergic state of the animals re-vaccinated with plague or anthrax monovaccines. However, re-vaccination with the tularemia and brucellosis monovaccines as well as with the trivaccine against plague, tularemia and brucellosis led to an increase in the number of animals reacting positively to tularin or brucellin as well as to more marked reactions against these allergens.

Agglutination tests made before and after re-vaccination showed in general that the re-vaccination led to an increase of the agglutinin production not only as far as the antigens used for re-vaccination were concerned, but also in the case of the other antigens. However, the authors stated,

"the anthrax antigen inhibited the immunizatory functions of the tularemia antigen and especially of the brucellosis antigen. This inhibition was very clearly manifested when the animals vaccinated with the combined (plague, tularemia, brucellosis and anthrax) vaccine were re-vaccinated with anthrax vaccine."

Summarizing the results of challenge tests made with (a) 200 DCL (200,000 organisms) of a virulent plague strain; (b) 1,000 DCL (5,000 organisms) of a virulent tularemia strain; (c) 2 infecting doses (10 organisms) of a Br. melitensis strain and (d) 10 DCL (280,000 spores) of Tsenkovskii's second vaccinal strain, the authors stated

"that the re-vaccination of the animals with one of the antigens composing the combined preparations used for vaccination did not lead to a lowering of the immunity against the other antigens. At the same time it is necessary to note that almost always there were more immune animals in the groups vaccinated and re-vaccinated with the monovaccines than among the animals receiving the combined preparations."

Tularemia-II/96

In their conclusions they also admitted that

"After re-vaccination of the animals with a vaccine composed of four antigens (plague, tularemia, brucellosis and anthrax) the latter (anthrax antigen) inhibited the production of an immunity against tularemia and plague."

On account of these observations, the two authors considered it undubitably possible to utilize the trivaccine against plague, tularemia and brucellosis for vaccination campaigns, regardless whether or not future re-vaccinations were envisaged. However, further studies were necessary to determine the role of the anthrax antigen in combined vaccination.

For a study of the duration of the immunity of guinea-pigs vaccinated combinedly against plague, tularemia, brucellosis and anthrax, Borodko and Samsonovich (1962) either injected their test animals subcutaneously with a vaccine mixture containing per dose of 1 ml one billion each of plague bacilli and brucellae as well as 330,000 tularemia bacilli and administered at the same time 2 drops of the STI anthrax vaccine by the cutaneous route or used only the trivaccine against plague, tularemia and brucellosis. Control tests were also made with the tularemia and brucellosis monovaccines.

Results of allergic and agglutination tests made one month and 6 months after the administration of these various vaccines are shown in the following two tables:

Kind of Vaccine	A l l e r g i c T e s t s							
	With Brucellin				With Tularin			
	After 1 month Tested	After 1 month Nega- tive	After 6 months Tested	After 6 months Nega- tive	After 1 month Tested	After 1 month Nega- tive	After 6 months Tested	After 6 months Nega- tive
Plague, tularemia brucellosis and anthrax	82	3	82	44	82	6	82	32
Plague, tularemia and brucellosis	87	5	87	48	87	5	87	24
Brucellosis mono- vaccine	21	2	21	14	-	-	-	-
Tularemia mono- vaccine	-	-	-	-	18	1	18	6

Tularemia-II/97

<u>Kind of Vaccine</u>	<u>Agglutination Tests</u>			
	<u>Brucellosis Antigen</u>		<u>Tularemia Antigen</u>	
	<u>Titers After</u>		<u>Titers After</u>	
	<u>1 Month</u>	<u>6 Months</u>	<u>1 Month</u>	<u>6 Months</u>
Plague, Tularemia, brucellosis and anthrax	1:108	1:33	1:127	1:29
Plague, tularemia and brucellosis	1:130	1:33	1:172	1:29
Brucellosis monovaccine	1:160	1:42	-	-
Tularemia monovaccine	-	-	1:197	1:43

Thus, the authors commented, after 6 months the test animals showed less frequently positive allergic reactions and lower agglutinin titers. The number of animals giving positive results in agglutination tests was also lower 6 months after immunization than after 1 month.

Results of challenge tests made after 1 month and 7 months with (a) 200 DCL of a virulent plague strain, respectively (b) 1,000 DCL of a tularemia strain; (c) 2 infecting units of a virulent Br. ovis strain and (d) 10 DCL of TSenkovskii's vaccinal anthrax strain No. 2 are shown in the following two tabulations:

<u>Kind of Vaccine</u>	<u>Results of Challenge Tests with</u>							
	<u>P. pestis</u>				<u>B. tularensis</u>			
	<u>After 1 Month</u>		<u>After 7 Months</u>		<u>After 1 Month</u>		<u>After 7 months</u>	
	<u>Tested</u>	<u>Succumbed</u>	<u>Tested</u>	<u>Succumbed</u>	<u>Tested</u>	<u>Succumbed</u>	<u>Tested</u>	<u>Succumbed</u>
Plague, tularemia, brucellosis and anthrax	6	0	5	2	6	1	7	3
Plague, tularemia and brucellosis	6	2	8	5	6	1	6	3
Plague monovaccine	6	4	10	3	-	-	-	-
Tularemia monovaccine	-	-	-	-	6	1	10	5

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<u>Kind of Vaccine</u>	<u>Br. ovis</u>				<u>B. anthracis</u>			
	<u>After 1 Month</u>	<u>General Infection</u>	<u>After 7 Months</u>	<u>General Infection</u>	<u>After 1 Month</u>	<u>Suc-cumbed</u>	<u>After 7 Months</u>	<u>Suc-cumbed</u>
Plague, tularemia, brucellosis and anthrax	6	0	5	2	6	3	5	3
Plague, tularemia and brucellosis	7	2	7	4	-	-	-	-
Brucellosis monovaccine	7	1	11	4	-	-	-	-
Anthrax monovaccine	-	-	-	-	6	3	10	7

Commenting upon these findings, the authors maintained that

"As a result of vaccinations with either the combined or the monovaccines the guinea-pigs proved highly resistant against the causative organisms of plague, brucellosis and tularemia, and less resistant against anthrax. 6-7 months after vaccination the immunity against plague, tularemia, brucellosis and anthrax became lowered, somewhat more markedly in the animals immunized with complex vaccines."

- (7) Tularemia, brucellosis and leptospirosis. - Gorchakova (1961) reported in a brief note on simultaneous vaccination of rabbits by various routes and in various dosages with live tularemia and brucellosis vaccines and with a penicillated vaccine prepared from the strain Leptospira grippotyphosa. Results obtained with this combined vaccine were identical with those in the animals protected with the corresponding monovaccines, thus showing that the combined vaccination did not lead to phenomena of inhibition.
- (8) Tularemia, brucellosis and virus encephalitis. - In order to explore the possibility of a combination of tularemia and brucellosis vaccination with that against tick-borne and Japanese encephalitis, Ryzhov (1962) experimented with rabbits,

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using besides live dry tularemia and brucellosis vaccines (prepared in the Gamaleia Institute and in the Kashintsev Biofabrika respectively) formolized anti-encephalitis vaccines.

The first group of 16 animals tested were given simultaneously 2 ml of the formolized vaccines subcutaneously and the tularemia and brucellosis vaccines by the cutaneous route; then two further doses of 3 and 4 ml of the encephalitis vaccines were administered within 10 days. Results of the combined vaccination were found to be identical with those obtained by the separate administration of the prophylactics concerned.

In a second series of tests groups of 4 rabbits were immunized respectively with (a) tick-borne encephalitis vaccine subcutaneously and tularemia vaccine cutaneously; (b) tick-borne encephalitis vaccine subcutaneously and brucellosis vaccine cutaneously; (c) Japanese encephalitis vaccine subcutaneously and tularemia vaccine cutaneously; (d) Japanese encephalitis vaccine subcutaneously and brucellosis vaccine cutaneously; (e) tularemia and brucellosis vaccines cutaneously and then 10 days later with the two anti-encephalitis vaccines administered subcutaneously; and (f) first with the two anti-encephalitis vaccines and 10 days later with the tularemia and brucellosis vaccines.

The results of immunization with these various combinations of vaccines did not materially differ from those obtained in the first series of tests. It was noted that immunization with the anti-encephalitis vaccines led to a state of para-allergy against the tularemia allergen.

- (9) Tularemia, intestinal infections and tetanus. - The possibility of combining anti-tularemia vaccination with the administration of the polyvaccine NIISI¹ seems to have been studied by Akimenko (1949). He concluded his short report, in which he also referred to experiments on guinea-pigs and

1. As summarized by Shpigunov (1959), the polyvaccine NIISI, introduced in 1941 by Aleksandrov and Gefen primarily for the armed forces, conferred protection against gastrointestinal infections (typhoid, paratyphoid A and B, Flexner and Sonne dysentery and cholera) and against tetanus.

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rabbits with combinations of tularemia and smallpox vaccines and of tularemia vaccine with tetanus anatoxin, by stating that

- "1. The combined vaccination simultaneously against tularemia, smallpox, tetanus and the group of intestinal infections is theoretically sound and practically adequate; one may also postulate the epidemiological efficacy of this combination of vaccines.
2. There develops a sufficiently high immunity against all components of these antigenic complexes.
3. This combination of vaccines is simply to use and gives the possibility of creating with the aid of one operation within a short time in the population a large stratum of immunity against the infections concerned, thus ensuring the possibility of a successful fight against the danger of infectious diseases.
4. The efficacy of this combined vaccination serves as a true proof for the possibility of creating simultaneously a specific insusceptibility to several infections; the immunity thus developing is the result of the presence in the immunized subjects of several kinds of immunity: symbiotic, sterile anti-bacterial, anti-virus and antitoxic."

Rogozin and Beliakov (1958), referring in their profound study on "The vaccinal process in combined vaccination" to the problem presently under review, stated that

"thus far very little has been published on the principles of the efficacy of the combined use of live and killed vaccines. Taking account of this fact, our co-workers made experimental studies on the laws of immunogenesis in animals immunized with tularemia vaccine, tetanus anatoxin and vaccines against intestinal infections. As the latter served tetravaccine, the polyvaccine NIISI and heat-killed typhoid and dysentery vaccines prepared in the laboratory (Li Li, 1956; Nikitin and Mishchenko, 1957¹)."

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1. No reference to the publication of these two workers could be found.

As Rogozin and Beliakov continued, various schemes of combined or successive immunization with these vaccines were used for studies on white mice, guinea-pigs and rabbits. For an evaluation of the results advantage was taken of agglutination tests, titrations of the protective and antitoxic properties of the sera of the test animals, allergic and challenge tests. The response to re-vaccination was likewise studied.

As a result of these investigations,

"no substantial difference could be detected in the level of immunological changes in the animals vaccinated with the above mentioned preparations separately or in combination (mixture) or in a complex manner, i. e. when the vaccines against intestinal infections and the tetanus anatoxin were administered subcutaneously and the tularemia vaccine at the same time by the cutaneous route. Successive immunization consisted of immunization of the test animals with the vaccines against intestinal infections combined with tetanus anatoxin, and of administration of tularemia vaccine at an interval of 35 days. In such tests also one could not observe a competition between the immunological processes: the immunological changes were similar to those observed in control animals individually immunized with the preparations under test and also in the animals of the above mentioned groups, immunized by the combined or complex methods."

To exemplify their observations, Rogozin and Beliakov quoted a table showing the agglutinin titers found in tests with tularemia antigen made at various intervals with the sera of guinea-pigs which had been immunized either separately or combinedly with tularemia vaccine and the NIISI polyvaccine:

<u>Method of</u> <u>Immunization</u>	<u>D a y s A f t e r I m m u n i z a t i o n</u>			
	<u>15</u>	<u>30</u>	<u>60</u> (revaccination)	<u>75</u>
Separately	1:100	1:120	1:120	1:120
Combinedly	1:100	1:100	1:140	1:120

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Thus, the two observers noted, after combined vaccination the agglutinin titers rose somewhat more slowly but were sixty days after immunization even higher than in the animals separately.

For the convenience of record reference is made also at the present juncture of the publications of two groups of workers who combined the administration of the tularemia and NIISI vaccines with that of still other prophylactics:

- (a) A preliminary report "On the immunological efficacy of simultaneous vaccination against tularemia and brucellosis and with polyvaccine NIISI" was rendered by Uzbekova and her assistants in 1959. This record, quoted by Uzbekova and her colleagues in their 1962 publication, could not be consulted.
- (b) Arslanova and a group of co-workers which included Rogozin and Beliakov, recorded in 1960 the results of observations made from 1956 to 1958 in persons immunized simultaneously with the NIISI, smallpox and plague vaccines and also, either at the same time or successively with tularemia vaccine.

In their first series of these tests the tularemia vaccine was given separately either before or after the combined administration of the other above mentioned prophylactics. The NIISI polyvaccine was injected into the back, the smallpox vaccine inoculated on the left arm. In one group of 120 people the plague vaccine was at the same time cutaneously administered on the right arm and this operation was repeated after 20-25 days. In another group of 140 people immunization was effected by intracutaneous injection. The mode and time of tularemia immunization is not stated.

While the reactions in the first group of 120 were not marked, the intracutaneous vaccination against plague led in 59 persons of the second group (41.2%) to quite severe local and general reactions with fever up to 39.5°C and involvement of the regional lymph nodes in 26%. Therefore no further use was made of this mode of vaccination during 1957 and 1958.

In order to evaluate the results of the combined vaccinations, the authors made in 1956 agglutination tests with sera taken from 50 of the immunized persons before vaccination and one and three months after it. It was found that

"After immunization with the combined vaccines the agglutinin titer rose at an average from 1:10 to 1:40 in the tests with typhoid, paratyphoid and dysentery antigens, and up to 1:50 in the tests with the tularemia and plague antigens (which gave a negative result before immunization)."

Agglutination tests made in 1957 and 1958 with typhoid, paratyphoid and dysentery antigens showed analogous rises of the agglutinin titers in the sera of combinedly vaccinated persons. Complement fixation tests made during this period in 103 of the immunized people gave a positive result with smallpox antigen in 9 instances and with plague antigen in 14.

The authors admitted in their conclusions that, as far as the serological tests went, the immunological changes produced by the combined vaccinations appeared to be inconsiderable.

- (10) Combined use of tularemia vaccine and anatoxins. - In addition to the observations on the combined use of tetanus anatoxin and tularemia vaccine referred to above, the possibility of using this vaccine simultaneously with anatoxins has been studied by Kovtanovich (1954) and by Saltikov and Zemskov (1960).

As quoted without a reference by Gubina (1957 a), the work of Kovtanovich showed

"that immunization of rabbits with the B. perfringens anatoxin together with live vaccines (tularemia and brucellosis) is accompanied by a more active production of antitoxin than is the case in immunization with the anatoxin alone. The state of immunity in regard to the brucellosis and tularemia infections has not been studied by the author."

Saltikov and Zemskov (1960) experimented on rabbits and guinea-pigs subcutaneously injected with an ex tempore prepared mixture of (a) a resuspended lyophilized penta-anatoxin which, adsorbed to aluminium hydroxide, included the tetanus, Botulinus A and B, B. perfringens and B. oedematiens anatoxins; (b) EV plague vaccine or NIEG tularemia vaccine (the latter in a dose of 25 million organisms).

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It was found that the combined administration of the penta-vaccine and either the plague or tularemia vaccine exerted no untoward influence on the antitoxin level in the serum of the test animals. Likewise, as shown by challenge tests 27 days after the combined immunization, the presence of the penta-anatoxin did not inhibit the protective action of the plague or tularemia vaccines.

- (11) Tularemia and pseudotuberculosis. - Olsuf'ev (1960), briefly discussing the problem of combined vaccination in the chapter on immunology of the work on tularemia edited by him and Rudnev, suggested that besides other live vaccines that against P. pseudotuberculosis ought to be used in combination with the tularemia vaccine.

- (12) Combination of tularemia vaccination with the administration of streptomycin. - Titova and Potapova (1957) experimented with groups of white mice which were subcutaneously injected with 1,000 organisms of Gaikii's vaccinal tularemia strain No. 15 and then, commencing at different intervals after the immunization, were given daily subcutaneous doses of streptomycin. As shown by the examination of sacrificed animals, the streptomycin-treated mice remained free from tularemia bacilli even if treatment with the antibiotic had been started six days after vaccination. Challenge of the mice left to survive with a virulent tularemia strain on the 21st day after vaccination gave the following results:

<u>Group of Mice</u>	<u>Time of Commencement of Streptomycin Treatment after Vaccination</u>	<u>Challenge Dose</u>	<u>Number of Mice</u>		
			<u>Tested</u>	<u>Died</u>	<u>Survived</u>
I	1 hour	1,000 organisms	5	5	-
II	2 days	"-	11	11	-
III	4 days	"-	10	10	-
IV	6 days	"-	5	3	2
V	Controls	"-	4	-	4

Commenting on their findings, the authors stated that

"The results show that the administration of streptomycin during the period of immunization prevents the development of an immunity. This may be ascribed to the chemotherapeutic action of streptomycin, impeding the multiplication of the vaccinal strain in the organism.

Thus one may consider this phenomenon as proof for the infectious character of the anti-tularemic immunity resulting from vaccination with an attenuated strain."