A FURTHER STUDY OF SPECIFIC SUBSTANCES OF THE TULAREMIA BACILLUS WHICH INDUCE A RAPID ALLERGIC RESPONSE

G. K. Shipitsina, R. A. Savel'eva, I. V. Rodionova, and L. S. Kolyadnitskaya

Department of Biochemistry (Head – Candidate of Biological Sciences V. A. Blagoveshchenskii) and Laboratory of Tularemia (Head – Professor N. G. Olsuf'ev) N. F. Gamaleya Institute of Epidemiology and Microbiology AMN SSSR, Moscow
(Presented by Active Member AMN SSSR N. N. Zhukov-Verezhnikov)
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At present, one of the best diagnostic tests for tularemia is made by applying "tularin" to the skin or injecting it intracutaneously, and it can also be used to determine the immunity of inoculated subjects. The allergic cutaneous reaction usually develops after one or two days.

I. N. Maiskii and G. K. Shipitsina [3] showed that a preparation ('tuallergen') from a virulent tularemia culture had a specific effect and could be used for the rapid diagnosis of tularemia. When injected intracutaneously into patients who were suffering or who had recovered from tularemia, or into subjects who had been inoculated with a live vaccine, it caused a local allergic response accompanied by hyperemia and infiltration to develop within 15 - 30 minutes.

Later, G. K. Shipsitsina showed that this preparation contains only a portion of the tularemic antigen complex; it is rich in the specific polysaccharide but poor in the lipid component. The whole antigen complex of virulent tularemia consists of a complex substance which on hydrolysis with weak acetic acid splits up into five fractions. I) protein; II) protein - polysaccharide - nucleic; III) protein - polysaccharide; IV) lipid; and V) a residue consisting of products of the partial hydrolysis of the original antigen. The characteristic feature of the antigen, due to components II and III is the large proportion of lipids, representing 34% of the whole [6, 7].

A. A. Kontorina [2] showed that the polysaccharide fraction separated by partial hydrolysis is a good substance for applying to the skin as a test for tularemic allergy, and is effective as an inoculation. The claim is made that the fraction is a polysaccharide, although this result is not borne out by its chemical properties [1]. The fact that no protein is obtained on acid hydrolysis is no proof that none is present, because stable polysaccharide-protein complexes form part of tularemia bacteria, and it is not possible to separate protein from them even by prolonged hydrolysis with 0·1N acetic acid or by the action of proteolytic enzymes [4, 5, 6].

In 1954, quite independently of A. A. Kontorina, we made a preparation from a virulent culture of the tularemia bacterium by a similar method. When given in dilutions of from 1:1000 to 1:100,000 to patients who had recovered from or who had been inoculated against tularemia, in most cases it had a specific action, producing necroses and side effects. A similar reaction was produced by an antigen separated from the culture by the action of trichloracetic acid. The necroses were due to the presence in the antigen of a large proportion of lipids. When fractions I and II, which contained no lipids, were applied to immune animals, the specific reaction occurred earlier, and as a rule there were no necroses [5].

The object of the present investigation has been to study the allergic activity of fractions II and III, separated from the antigen of the virulent strain (No. 503) of the tularemia organism, when applied to subjects who had recovered from tularemia or who had been vaccinated against it; comparative tests were also made of the allergic activity of antigens obtained by different methods from virulent strain (No. 503) and from an attenuated vaccine.

METHODS

The vaccine strain was grown in an aerated fluid medium. The allergic activity was studied in terms of the chemical constitution of the antigens and the amounts of the different fractions obtained by acid hydrolysis (Table 1).

TABLE 1. Chemical Constitution and Amount of the Different Fractions in Antigens of the Tularemia Organism of Virulent and of Vaccine Strains

Antigen strains	Chemical constitution of the preparations (in percent)					Fractions (percentage)				
	total nitrogen	total phosphorus	reducing substances	Hexosamine	ash	I	II	III	IV	V
Virulent strain No. 503	6,24	1,48	27,63	10,09	8,75	6,82	33,59	15,51	33,35	14,52
Vaccine strain (reduced)	1,85	0,80	81,31	1,85	9,35	1,68	82,32	2,24	3,61	14,33

* After hydrolysis (action on glucose).

The antigen of the vaccine culture showed an extremely high carbohydrate component (81% of reducing substances) and a very small amount of substances containing nitrogen and phosphorus. Fraction IV (lipids) was ten times smaller than in the virulent strain, and fractions I and III were much smaller too. Fractions II comprising 82.32% of the complex consists almost entirely of the pure specific polysaccharide (92.1% reducing sugars, 1.05% total nitrogen).

The allergic activity of fractions II and III of the antigen of strain No. 503 was tested on 8 subjects who had recovered from tularemia, and on 9 subjects who had been vaccinated against it. The preparations were diluted 1:1,000, 1:5,000, 1:10,000, 1:25,000, and 1:50,000 times and 0 1 ml of the diluted fluid was injected strictly intracutaneously into the forearm.

The reaction to all the injections took the form of a marked hyperemia extending to a circle of not less than 1 cm in diameter around the papule, which developed in all except one of the subjects who had had the disease, as a rule within 15 minutes. In one case, the reaction was doubtful after injecting fraction II in a dilution of 1:50,000, and the hyperemia extended over an area only 0.4 - 0.5 cm in diameter after 30 minutes. After 24 and 48 hours, the reaction had either ceased entirely, or only traces of hyperemia and infiltration remained 0.3 cm in diameter). At a dilution of 1:1,000, the marked reaction was maintained for 24 hours or more, but just as with smaller concentrations, there were no side effects. In all cases, the specificity of the effect was confirmed by simultaneously injecting tularin, and by the absence of any reaction in nonsensitized subjects.

The results show that to carry out an allergy test in subjects who have recovered from the disease, fractions II and III of antigen strain No. 503 diluted 1:10,000 or more may be used. It seems to us that it would be useful to make a further study of these fractions to determine their usefulness for the rapid diagnosis of the condition in human subjects.

In studying the effect of fraction II diluted 1:10,000, and 1:25,000 times on subjects who had been vaccinated against tularemia, it was shown that there was a reaction in those who had been vaccinated not more than 6 months previously (subjects D., M., K., S., M., B.,). Subjects who had been immunized from 15 months to 8 years previously, either did not react to the injection even at a concentration of 1:1,000, or else there was a doubtful response. This difference can probably be attributed to the different degrees of sensitization in subjects which have recovered from the disease or have been vaccinated against it at various times before the test. The results of the effect of fraction II of the virulent strain No. 503 on innoculated subjects must be regarded as preliminary trials.

Comparative tests of the allergic properties of the antigens obtained from the vaccine and from the virulent strains were made on 96 guinea-pigs having a maximal immunity. This immunity was established by vaccinating with live tularemia vaccine followed by infection with the virulent culture. The very small amount of lipids in the antigen of the vaccine strain ensured the production of marked allergic responses without any side effects.

The separation of the antigens was effected by several methods: that of Boiven [7] was applied to the acetone-dried bacterial mass of the virulent and vaccine strains, and that of Larson [8] to the similarly dried product of the virulent strain; Boiven's method was used on a suspension of live bacteria of vaccine strain which had been disintegrated by ultra-sound, and by salting-out with 40% ammonium sulphate from the same suspension. To increase the yield of antigen, 10 w/cm^2 of ultra-sound was applied to the bacterial suspension for two hours, at a frequency of 1200 kc.

The antigens were tested in dilutions from 1:1,000 to 1:500,000. Each animal received several dilutions of the antigen simultaneously. Reactions occurred 1, 3, 6, 24 and 48 hours after the intracutaneous injection of 0.1 ml of the preparation. The activity of the antigen could be eliminated by autoclaving for 30 minutes at 1 atmosphere. A control test of the preparations was made with tularin, and both substances were injected simultaneously into nonimmune guinea-pigs (Table 2).

TABLE 2. Allergic Response in Guinea-pigs Immunized against Tularemia to the Injection of Antigens produced from Vaccine and from Virulent Strains of the Tularemia Organism

Strain	Method of obtaining the antigen		Total number of guinea pigs	Dilution of antigen 1:1000 +	1:1000 ±	1:10 000 +	1:10 000 ±	1:25 000 +	1:25 000 ±	1:50 000 +	1:50 000 ±	1:100 000 +	1:100 000 ±	1:500 000 +	1:500 000 ±
Virulent strain No. 503	Boiven's method		12	12/12	0	11/12	1/12	No tests				0	12/12	0	5/12
	Larson's method		12	12/12	0	11/12	1/12					0	7/12	0	0
Reduced vaccine strain	Boiven	Not autoclaved	29	11/12	1/12	19/29	8/29	14/17	0	11/17	2/17	2/12	8/12	0	1/12
		Autoclaved	23	No tests		17/23	6/23	13/23	10/23	9/23	13/23				
	Bacteria treated with ultra-sound	Boiven	10	10/10	0			6/10	4/10	2/10	8/10	No tests			
		Salted-out with ammonium sulphate	10	10/10	0	No tests		7/10	3/10	5/10	5/10				

Note: The numerator represents the number of animals which reacted and the denominator the number used in the particular experiment; + indicates a positive reaction (appearance of hyperemia and infiltration for 1 cm or more within 6 hours); ± indicates doubtful reaction (appearance of a mild hyperemia and infiltration of less than 1 cm in diameter.

In the reactions to the antigen from the virulent strain diluted 1:1,000, and in some animals when diluted 1:10,000, a hemorrhage occurred in the center of the papule after 3 - 6 hours, and necrotic areas had developed after 24 hours.

The results indicate that there is no essential difference in the response of guinea-pigs to the injection of antigens of the virulent or the vaccine strain, when diluted 1:1,000. However, in nine of the ten animals, a positive reaction to the antigen from the virulent strain occurred after only one hour, and as a rule there were necroses, whereas to the antigen from the vaccine strain the reaction developed more slowly, and was not well marked until six hours

had passed, and only occasionally did any necroses develop. Further, in this case the number of animals which did not react until 24 hours had passed was greater, the response was doubtful, and in some there was no reaction at all. Neither antigen produces any response in dilutions of 1:100,000 and 1:500,000. No effect on the response was found to accrue from the different methods of extracting the antigens from the virulent strain.

Autoclaving the antigens of the vaccine strain produced no essential difference in their activity. The small difference that did occur could be attributed to the different numbers of animals in the groups, and to individual differences. The strict specificity of all the preparations tested was confirmed by the fact that the reactions that they elicited were precisely the same as those induced by tularin in the same animals. In all cases, control experiments on nonimmune guinea-pigs gave negative results.

When injections were given of antigens from a vaccine strain exposed to ultra-sound and separated either by Boiven's method or by salting-out with ammonium sulphate, there was no definite effect for the first six hours, though an intense reaction occurred after 24 or 48 hours. The breaking up of the culture by ultra-sound therefore reduces the activity of the preparations.

The investigations have shown that the antigen of the vaccine strain may be used as a specific agent for eliciting a rapid allergic response. The negligible lipid fraction has the effect that the preparation produces a weaker reaction than does that from a virulent strain, and makes it unnecessary to carry out complex fractionation.

To follow these results on guinea-pigs, the next step is to test the allergic activity of the antigen of the vaccine strain on human subjects, and it will be reported in a separate communication.

SUMMARY

It was found that fractions II and III (obtained by mild acid hydrolysis of the antigen of a virulent Pasteurella tularensis culture) diluted 10,000 or more times could be used to test for the allergic response in subjects who had had tularemia. A specific allergic reaction was provoked within 15 - 30 minutes at the site of administration. A comparative study of the allergic response of guinea-pigs was investigated by means of the following preparation: antigens isolated from the bacterial mass of the virulent and vaccine strains (dried by acetone) by either Boiven's or Larson's method, an antigen isolated by Boiven's method from a suspension of living bacteria of the vaccine strain which had first been disintegrated by treatment with ultra-sound at a frequency of 1200 kc and 10 w/cm^2, and an antigen obtained from the same suspension by salting-out with 40% ammonium sulphate solution. The antigens isolated by the different methods from the virulent culture produced identical responses. The antigen from the vaccine strain produced a slower response, which was not pronounced until six hours had passed. It may, however, be used in human subjects in whom it causes an allergic reaction which is rapid in comparison with that elicited by tularin, and owing to the insignificant lipid fraction there are no side effects. Sterilization by autoclaving at one atmosphere pressure did not reduce the activity of the preparation. Disintegration of the bacterial suspension by ultra-sound reduced the allergenic activity of the antigen isolated from it.

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