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Studies on the Specificity of Anthrax Precipitin Serum

(10) The Antigenic Relation between *Bacillus anthracis* and other Bacterial Species which Exhibit Group Reaction with Anti-anthrax Serum

By

Shizuo TAKAGI and Takeshi HIRONAO

Department of Veterinary Science, College of Agriculture

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A series of experiments on the specificity of anthrax precipitin serum were made. Experimental results in diverse cases of group reaction exhibited by extracts of various bacterial species with anti-anthrax serum have been reported.[1-7] The present report presents the results in converse cases (*i.e.*, the precipitin reaction of the anthrax extract with heterologous antisera), and the results of the cross-reaction test for the relation between antigenic structures of these bacterial species. It was, as an interesting fact, reported in the previous paper[8] that in the extracellular product of the anthrax bacillus grown on agar medium a substance seems to be contained, which itself exhibits a very weak antigenicity *in vivo*, but which, when mixed with bacterial cells, seems to be capable of inciting the production of anti-body, and a substance which reacts strongly with anti-anthrax serum. The present report also includes results of the test for the presence of such antigenically active substances in products of other bacterial species, and results of the test for the antigenicity of these products by the same experimental method as for the bacterial extracts, and results of the test for the relation between antigenic substances contained in extracts and in products by the absorption technique. The results are presented below.

Materials and Methods

1. **Organisms.** The following organisms were used. Details of their sources have been described in our first paper.

B. anthracis
B. 21
B. cereus
B. subtilis
Micrococcus pyogenes var. *aureus* (Abbrev. *M. aureus*)

2. **Bacterial extracts.** The extraction procedure is essentially similar to that described previously. Briefly as follows:—

a. **Agar culture extracts.** Cultures of each organism on nutrient agar (at 37°C. for 8 to 10 hrs.; *M. aureus*, for 18 to 20 hrs.) were collected, and without washing, autoclaved, dried and ground in a mortar. Extraction was made from the powder in the proportion of 10 mg. per cc. saline.

b. **Broth culture extracts.** Cultures of each organism in nutrient broth (at 37°C. for 18 to 20 hrs.; *B. anthracis*, for 65 to 70 hrs.) were collected by centrifugation, washed with saline 3 times, autoclaved, dried and ground in a mortar. Extraction was made in the same manner as for the agar culture.

3. **Antigenically active substances in bacterial products.** Cultures of each organism on agar slant (in medium-sized test tubes, approximately 15×170mm., at 37°C. for 12 to 14 hrs.) were collected, suspended in saline in the proportion of cells on one slant per cc., shaken vigorously for several minutes, centrifuged and the supernatant fluid was filtered through a Seitz disk to remove bacterial cells completely. This cell-free filtrate, a solution containing a bacterial product, which will hereafter be described as a "product", was tested for its antigenic activity.

4. **Antisera.** Antisera were prepared by the same method as for the anti-anthrax serum described in our first paper. Briefly, young cultures of each organism were collected, suspended in 0.5 per cent formalin saline, with which rabbits were immunized.

5. **Absorbed sera.** Absorbed sera were prepared as follows:—

Appropriate volumes of each serum to be absorbed were mixed with appropriate volumes of each absorbent (bacterial extract or product) which had previously been concentrated to an appropriate volume in order to complete absorption. The serum-absorbent mixture was allowed to stand at room temperature for 3 hours, then centrifuged and the supernatant fluid was used as an absorbed serum.

6. **The precipitin reaction.** The test was performed by the same method as described in the previous paper.

Results

1. **The cross-reaction with bacterial extracts.** In cases in which both an organism which may contain both agar substance and extracellular product and a broth culture extract of the same organism which is presumed to be free from such extracellular substances were used as antigens, significant antigenic differences were not observed between these two extracts of the same organism, with the exception of *B. anthracis* which exhibited a slight difference in antigenic activity. The extract of each organism reacted to the highest degree with its own antiserum, but it also reacted with heterologous antisera, and no specific extracts were obtained, except the extract of the anthrax bacillus broth culture. The extract of *M. aureus*, particularly, reacted with almost all the antisera prepared against organisms of the genus *Bacillus*. It is interesting that the *B. 21* extract reacted strongly with the antiserum prepared against *B. anthracis*, but the converse reaction scarcely occured and the extract of *M. aureus* reacted with heterologous antisera, but with the anti-*aureus* serum none of the heterologous extracts, except that of *B. subtilis*, reacted. In each of *B. 21* and *M. aureus*, the specificity of its antiserum

Table 1. The cross-reaction with bacterial extracts.

Extract \ Antiserum		Anthrax serum	B. 21 serum	Cereus serum	Subtilis serum	Aureus serum
Agar culture	*B. anthracis*	4	0	1	1	0
	B. 21	3(4)	3	1	0	0
	B. cereus	0(1)	1	4	1	0
	B. subtilis	1(3, 0)	1	2	4	2
	M. aureus	0(1, 2)	1	2	2	2
Broth culture (Washed)	*B. anthracis*	4	0	0	0	0
	B. 21	3	4	1	1	0
	B. cereus	0	1	4	2	0
	B. subtilis	0(1)	1	2	3	1
	M. aureus	0(1)	1	2	2	3

4~0 : The degree of reaction.
( ) : An exceptional degree of reaction exhibited by some antisera.

and the specificity of its extract seemed to differ each other.

2. **The cross-reaction with products.** With bacterial products, similar tests were made by the same procedure as applied in experiment 1. As shown in Table 2, in the product of each organism of the genus *Bacillus*, the presence of a substance which is antigenically active against its homologous antiserum was recognized. Such a substance, however, was not detected in the product of *M. aureus*. it is interesting that these active substances reacted specifically with their own antisera, exhibiting extreme specificity in comparison with the specificity observed in the case of extracts and with these substances no group reaction was observed between bacterial species. The product of *B. 21*, however, was exceptional and it exhibited, though the converse reaction did not occur, a group reaction with anti-anthrax serum. In the product of *M. aureus*, even a substance which may react with its own antiserum was not present.

Table 2. The cross-reaction with extracellular products.

Antiserum / Product	Anthrax serum	B. 21 serum	Cereus serum	Subtilis serum	Aureus serum
B. anthracis	4	0	0	0	0
B. 21	2	2	0	0	0
B. cereus	0	0	2	0	0
B. subtilis	0	0	0	3	0
M. aureus	0	0	0	0	0

3. **The relation between antigenic substances in the extract and in the product of each organism.** From the results of experiment 2, the presence of antigenic substances in products of agar-grown organisms of the genus *Bacillus*, which reacted with their own antisera in the manner somewhat similar to that in the case of extracts, was recognized and absorption tests were made to examine the homology of these antigenic substances contained in the extract and in the product of each organism. As shown in Table 3, when each

Table 3. Antigenic relation between active substances in the extract and product.

Serum absorbed / Absorbent \ Antigen		Anthrax		B. 21		Cereus		Subtilis	
		Product	Extract	Product	Extract	Product	Extract	Product	Extract
Anti-anthrax serum	Anthrax product	0	1						
	Anthrax extract	0	0						
Anti-B. 21 serum	B. 21 product			0	1				
	B. 21 extract			0	0				
Anti-cereus serum	Cereus product					0	0(1)		
	Cereus extract					0	0		
Anti-subtilis serum	Subtilis product							0	3
	Subtilis extract							0	0

serum was absorbed by the product and extract, respectively, precipitin activity of the serum against the extract and the product was removed in the case of absorption by the extract, but in the case of the product, precipitin activity against the extract, though fairly diminished, remained. From these results, antigenic substances in both extract and product seemed to share a relatively similar structure, though not completely identical. It seems that another antigenic substance is present in the extract, which is somewhat different from the substance contained in the product.

4. **The antigenic relation between active substances in products of B. anthracis and B. 21.** From the results of experiment 2, a common antigenic substance seemed to be present in both products of *B. anthracis* and *B. 21.* The antigenic relation was pursued

Table 4. Antigenic relation between products of *B. anthracis* and *B. 21.*

Serum absorbed \ Antigen / Absorbent		Anthrax product	B. 21 product
Anti-anthrax serum	B. 21 product	1	0
	Anthrax product	0	0

by the absorption test. As shown in Table 4, the anthrax product contained not only a substance which is common to the product of *B. 21*, but also a substance which is specific for itself, and this property was similar to that observed in the case of the extract.

Summary and Conclusion

It seems that most of the work on the antigenic structure of the members of the *Bacillus* group, especially that carried out by the precipitation method, has been performed with a view to separating *B. anthracis* from the other members of the group. SCHUTZ and PFEILER[9] and PFEILER and DRESCHER[10] have studied the antigenicity of extracts of *B. pseudoanthracis*, *B. mesentericus*, etc., against anti-anthrax serum, while ZOZAYA[11] has studied the antigenicity of polysaccharides of *B. anthracis*, *B. subtilis*, *B. mesentericus*, etc., against anti-anthrax serum. SIEVERS and ZETTENBERG[12] have tested for the cross-reaction of extracts of *B. subtilis*, *B. mycoides*, *B. mesentericus*, *B. vulgatus* and *B. cereus*, with each of their own antisera. LAMANNA,[13-15] working with spore extracts and anti-spore sera prepared against spore antigen, has separated *B. subtilis*, *B. vulgatus*, *B. mesentericus*, etc., from each other. The antigenic relation between *Micrococcus* and the members of the genus *Bacillus* has been studied only by SCHUTZ and PFEILER, who worked with *B. anthracis* and *Micrococcus*, and by the present senior author, who has worked on the antigenicity of *Micrococcus* against anti-anthrax serum. Other reports on the antigenic relation of *Micrococcus* to the other members of the *Bacillus* group are almost unavailable. Moreover, most of the work mentioned above has been carried out with the intracellur antigenic substances, and the work with extracellular products is scarcely known. Therefore, antigenic structures of *B. anthracis* and the other members of the *Bacillus* group—*B. 21*, *B. cereus* and *B. subtilis*—which exhibit a group reaction with anti-anthrax serum, and of *M. aureus* were studied by the use of their extracts and products.

Results are summarized as follows:—

1. In the case of extracts, significant antigenic differences were not observed between the agar culture extract of an organism which may contain agar substance and extracellular product and the broth culture extract of the same organism which may be free from such extracellular substances. A certain organism, however, exhibited a slight specificity with its broth culture extract. Every organism reacted to the highest degree with its own antiserum, but it also exhibited to some degree a group reaction with each other. Some organisms, however, exhibited a group reaction which is not convertible (*B. anthracis* and *B. 21*, and *M. aureus* and other organisms tested).

2. In the case of products, just as observed in the case of extracts, the presence of an antigenic substance which reacted to the highest degree with its own antiserum was recognized in the product of each organism tested, except *M. aureus*. It is interesting to note that the product was very specific in antigenicity in comparison with the extract (the *B. 21* product, however, exhibited a group reaction with anti-anthrax serum, but not convesrely). From this fact, the group reaction between the organisms seems to be due to the intracellular substances and therefore, it may be more available in certain bacteria to use their products for studying specificity of antisera than to use their extracts.

3. From the results obtained by the absorption test, the antigenic substances in extracts

seem to be common to those in the products, but the extracts seem to contain the other antigenic substances which are different from those in the products.

4. The product of the anthrax bacillus seemed to contain not only antigenic substances which are common to *B. 21*, but also substances which are specific for itself. The antigenic substance in a filtrate of certain agar-grown bacteria has been described in this report briefly as a "product". The validity of this designation seems to be uncertain, since it is thought that destructed bacterial cell components might be contained in the filtrate. It is thought, however, that such intence destruction will not occur in young, 12 to 14 hour cultures, and the designation "product" was applied for convenience sake.

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