COMPARATIVE STUDY OF THE AGGLUTINOGENS OF THE ENDOSPORES OF *BACILLUS ANTHRACIS* AND *BACILLUS CEREUS*[1]

CARL LAMANNA AND DANIEL EISLER

The Naval Biological Laboratory, School of Public Health, University of California, Berkeley, California

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Potentially, antigen-antibody reactions are a means for the specific identification of species. However, no such means are presently available to distinguish between cultures of *Bacillus anthracis* and *Bacillus cereus* (Brown *et al.*, 1958 *a*, *b*). The serological investigations conducted have not exhausted all the possibilities for trial. Only serological studies comparing the vegetative phases of *B. cereus* and *B. anthracis* have been recorded. Bacterial endospores possess antigens distinct from those of parent vegetative organisms, and it has been shown that the antigenic structure of bacterial endospores may be sufficiently specific to have taxonomic value (Lamanna, 1940*a*, *b*). For these reasons we have undertaken a comparative study of agglutinins against spores of strains of *B. anthracis* and *B. cereus* to learn whether or not these antibodies can provide for a means for differentiation between the two organisms.

MATERIALS AND METHODS

Strains. From Fort Detrick 20 strains of *B. anthracis* of varied origins were obtained. Of these, 7 strains were represented by both a culture of a smooth and a rough variant of quite different virulence (table 4). In addition, the veterinary spore vaccine strain Carbozoo (Lederle) was studied. All strains were nonmotile, an important characteristic of *B. anthracis* for cultural differentiation from *B. cereus* (Sterne and Proom, 1957).

Dr. K. L. Burdon kindly supplied 8 strains, Dr. Ruth E. Gordon 5 strains, and Dr. E. A. Steinhaus 6 strains of *B. cereus*. We independently confirmed their authenticity using the criteria of Smith *et al.* (1952).

Preparation of spore suspensions. Inoculum for production of spore crops was prepared by suspension of sporulated cultures in 0.85 per cent NaCl solution. Often it was desirable to insure that the inoculum was composed largely, if not exclusively, of spores by heat shocking the inoculum suspension for 7 min at 56 C, or 5 min at 60 C. For spore crops the surface of agar in flat wine bottles was inoculated and incubated for at least 2 weeks at 30 C for *B. cereus* and 35 C for *B. anthracis*. The medium for the crops was composed of 1.75 per cent agar, 0.5 per cent peptone, 0.25 per cent yeast extract, 0.125 per cent dextrose, 0.01 per cent K_2HPO_4, 0.0025 per cent $CaCl_2$, 0.001 per cent $MgSO_4 \cdot 7H_2O$, 0.001 per cent $MnSO_4 \cdot H_2O$.

Growths were harvested in sterile saline. The spores were recovered by centrifugation and washed 3 or more times by centrifugation from saline. Microscopic examinations of wet or stained smears were made to gauge the extent of contamination with vegetative forms. Preparations with vegetative organisms were discarded. Of course, this does not insure that the spore suspensions were absolutely free of vegetative cells and debris. The spore antigen suspensions were not treated in any way, such as adding a preservative, that knowingly would kill the spores. This was desirable since our interest was to study as closely as possible the natural state of endospore antigens.

Vegetative organisms suspension. B. cereus was grown at 30 C overnight, or for not more than a day, on agar slants. *B. anthracis* was grown in broth cultures aerated by shaking at 37 C.

Preparation of antisera. The turbidity of the antigen suspensions employed was equivalent to the no. 9 tube of a MacFarland nephelometer. With *B. cereus* spores a series of alternating (0.5 and 1.0 ml) subcutaneous and intravenous injections were given with varying rest periods between each series until a satisfactory agglutinating serum was obtained. With *B. anthracis*, 8 weekly (2 subcutaneous, 6 intravenous) 0.5-ml injections were given. New Zealand, Dutch, and

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TABLE 1

Homologous spore and vegetative organism agglutinin titers of rabbit antispore serum before and after adsorption with homologous vegetative organisms

Strain	Serum No.*	Antiserum Titers			
		Before adsorption		After adsorption	
		Spore	Vegetative	Spore	Vegetative
B. cereus					
NRS201	60NZW	1024		1024	<8
Ba2(19)	61NZW	1024	<4	1024	<8
NRS847	62NZW	SA	32	SA	8
NRS1256	67ANZW	1024	<8	1024	<4
NRS617	17,21NZW	2048	16	1024	<8
	10NZW	4096	<8	>2048	<8
	9,10D	4096	<8	4096	<8
Ba2(16)	11,12NZW	4096	SA	4096	SA
	11,12D	4096	SA	2048	SA
Ba2(22)	13NZW	1024	8	1024	64
	13,14D	2048	8	2048	32
Ba2(23)	15,16NZW	1024	SA	1024	SA
	15,16D	2048	SA	2048	SA
NRS793	1,2NZW	2048	<2	2048	<8
	1D	4096	<2	4096	<8
NRS946	4NZW	SA	8	SA	<8
	3,4D	SA	4	SA	<8
NRS232	5,6NZW	4096	2	>4096	32
	5,6D	4096	4	>4096	<8
NRS244	7,8NZW	2048	16	>4096	32
	7D			1024	<8
B. anthracis					
Carbozoo	8NZW	2560		640	<20
	9NZW	2560		5120	<20
381	4NZW	2560	80	320	320
	53NZW	2560	40	320	<20
	64NZW	160	<20	40	<20
	76NZW	1280	160	160	<20
384S	56NZW	1280	>20	<20	<20
	67Cal	1280	20	640	<20
	68Cal	1280	<20	640	<20
	69NZW	1280	<20	80	<20
385R	17NZW	640	SA	2560	<20
390S	30NZW	2560	<20	2560	<20
	58NZW	1280	<20	1280	SA
	59NZW	1280	<20	640	<20
	60NZW	80	<20	640	<20
	62NZW	640	<20	640	<20
	70Cal	320	<20	640	<20
	71Cal	1280	<20	640	<20
	73Cal	1280	<20	320	<20
	75Cal	640	<20	640	<20
B. anthracis					
391R	16NZW	320	1280	320	SA
	17NZW	320	1280	1280	SA
	18NZW	1280	2560	640	SA
394S	32NZW	640	<20	320	<20
	33NZW	640	<20	320	<20
	34NZW	640	<20	320	<20
395R	37NZW	640	160	80	<20
	38NZW	640	<20	320	<20
	42NZW	640	80	2560	<20
399S	19NZW	±5120	320	5120	<20
400R	45NZW	2560	SA	2560	<20
	46NZW	640	<20	1280	<20
	48NZW	2560	<20	2560	<20

< Refers to a negative reading at this lowest dilution tested; > refers to a positive reading at this highest dilution tested; SA = spontaneous agglutination.

* NZW refers to New Zealand White, D to Dutch, and Cal to California breeds of rabbits. Each number represents serum from an individual rabbit.

California breeds of rabbits were employed. Sera obtained before immunization did not agglutinate vegetative bacilli or endospores. To prevent death from anthrax, 4 weekly injections of 150,000 units of Penicillin G Procaine Crystalline (Lederle) were given to each rabbit concomitant with the spore injections. The procedure was followed not only with the hope of protecting the animals, but to prevent germination of spores (Curran and Evans, 1946) and infection with probable formation of specific vegetative state antibody. Still another group of animals was injected (once subcutaneously and 3 times intravenously) with $\frac{1}{10}$ the spore concentration listed above and given penicillin daily. Considerable mortality resulted in all the groups.

Sera were collected from 7 to 14 days after the last injection of antigen and stored near 0 C, or in a freezer at −20 C. As a preservative, merthiolate was employed in a final concentration of 1 to 10,000.

Agglutination reactions. As found in the past (Lamanna, 1940*a*, *b*), the traditional agglutination procedures proved inadequate for measuring spore agglutinin. Maximal titers were obtained with spore suspensions made up to an optical density of 0.6 as measured in a Coleman Jr. spectrophotometer at 640 mμ, mixed in 0.1-ml quantities with 0.1 ml of 2-fold serially diluted antiserum, and then shaken vigorously for 30 to 60 min at room temperature. It was desirable for ease in reading to add 0.8 ml of saline per tube after the shaking was completed. One reading was made immediately after the shaking, and usually a second reading was taken after overnight storage in a refrigerator. The latter observations were often more intense in the appearance of agglutination, resulting in one or several tubes higher positive readings. Prozones were encountered frequently (Lamanna, 1940*a*). Spontaneous agglutination was also noted with the endospores. Some strains of bacilli were more notorious offenders than others in this regard. An uninvestigated feature of spontaneous agglutination was its irregular occurrence among spore crops of a given strain cultivated at different times. Another annoying feature of spore

TABLE 2

Endospore agglutination tests with heterologous strains of Bacillus cereus and Bacillus anthracis employing antisera against B. cereus spores adsorbed with homologous strain vegetative organisms. Results recorded as number of strains giving the indicated titer.

Antiserum Strain and Rabbits	*B. cereus* Strains					*B. anthracis* Strains					Homologous Strain Titer
	Total no.	Titer				Total no.	Titer				
		< 8	8–64	128–512	1024+		<8	8–64	128–512	1024+	
NRS201 (NZW60)..	17	5	1	1	10	19	8	2	3	6	1024
Ba2(19) (NZW61)...	17	0	1	4	12	20	10	1	6	3	1024
NRS847 (NZW12)..	17	0	10	4	3	18	7	5	0	6	SA
NRS1256 (NZW67A).....	17	1	3	7	6	19	6	8	0	5	1024
NRS617 (NZW10).....	17	7	2	1	7	17	8	3	1	5	2048
(NZW17, 21).....	17	5	2	6	4	20	10	1	1	8	1024
(D9, 10).....	17	1	6	5	5	19	8	2	2	7	4096
Ba2(16) (NZW11, 12).....	17	1	2	2	12	19	7	4	5	3	4096
(D11, 12).....	17	0	2	5	10	20	8	5	3	4	2048
Ba2(22) (NZW13).....	17	1	9	6	1	20	12	1	3	4	1024
(D13, 14).....	17	1	9	5	2	21	10	5	1	5	2048
Ba2(23) (NZW15, 16)	17	3	1	6	7	20	9	1	2	8	1024
(D15, 16).....	17	1	2	2	12	20	8	0	2	10	2048
NRS793 (NZW1, 2).....	17	6	2	4	5	18	8	3	2	5	2048
(D1).....	17	3	3	2	9	18	6	1	3	8	4096
NRS946 (NZW4).....	17	6	8	3	0	18	6	8	1	3	SA
(D3, 4).....	17	4	3	8	2	20	10	0	5	5	SA
NRS232 (NZW5, 6).....	17	0	2	1	14	19	11	1	3	4	4096
(D5, 6).....	17	0	0	4	13	19	10	1	6	2	4096
NRS244 (NZW7, 8).....	17	0	3	2	12	19	8	4	4	3	4096
(D7).....	17	0	2	2	13	18	6	5	4	3	1024

agglutination studies is the tendency of the spores to be trapped in the liquid-air interface and to climb up the wall of the tube in a film of liquid which creeps up the wall of the tube away from the meniscus. This phenomenon is limited to salt solutions, and solutions with the most diluted quantities of antiserum, and suggests the phenomenon is a reflection of a relatively hydrophobic quality at the spore surface.

Before testing for heterologous endospore agglutinins, the spore antisera were adsorbed with homologous vegetative organisms to insure that agglutinations observed were specific for endospore agglutinins.

RESULTS AND DISCUSSION

Endospore antisera did not agglutinate vegetative organisms, or did so in low titer relative to spores. Vegetative *B. cereus* injected into rabbits resulted in either none or low titer agglutinins against endospores which were readily and and specifically removable by adsorption with homologous strain spores.

In table 1 are recorded agglutinin titers for homologous strain spores and vegatative organisms tested against the antispore sera prepared. The agglutination tests were done with the antisera both before and after adsorption with vegetative bacilli. The data indicate the existence of endospore specific agglutinins and provide the rational factual basis for the comparative study of spores of *B. anthracis* and *B. cereus*. Note the unexpected finding with 4 *B. cereus* and 1 *B. anthracis* spore antisera of a low titer rise in vegetative cell agglutinin *after* adsorption with vegetative organisms. In these cases it is possible that the attempted adsorption incompletely removed a vegetative bacillus agglutinin while eliminating a vegetative cell agglutination inhibitor.

Tables 2 and 3 record the spore agglutinin titers for the strains studied and demonstrate a widespread degree of cross reactions between the *B. cereus* and *B. anthracis* strains. The sera cannot be employed to distinguish between the two organisms, this in spite of the fact that in many cases agglutination titers were lower for the heterologous than homologous species.

An antianthrax serum prepared in the horse by injection of Sterne's spore vaccine (Weybridge strain) made available by the Microbiological

TABLE 3

Endospore agglutination tests with heterologous strains of Bacillus cereus and Bacillus anthracis employing antisera against B. anthracis spores adsorbed with homologous strain vegetative organisms. Results recorded as number of strains giving the indicated titer.

Antiserum Strain and Rabbits	*B. cereus* Strains					*B. anthracis* Strains					Homologous Strain Titer
	Total no.	Titer				Total no.	Titer				
		<8	8–64	128–512	1024+		<8	8–64	128–512	1024+	
Carbozoo (NZW8, 9)	16	8	5	3	0	20	8	0	2	10	2048
399 (NZW19)	17	10	2	4	1	18	14	2	2	0	SA
391 (NZW16, 17, 18)	17	7	10	0	0	20	7	2	0	11	1024
381 (NZW53, 76)	16	9	7	0	0	14	8	1	4	1	256
400 (NZW45, 46, 48)	16	4	7	4	1	18	0	12	5	1	1280
395 (NZW38, 42)	17	6	9	2	0	17	3	6	6	2	SA
394 (NZW32, 33, 34)	17	13	4	0	0	18	11	1	3	3	2048
390 (NZW30, 58, 59, 63)	17	7	8	2	0	18	10	0	1	7	1024
(CAL70, 71, 75)	17	9	6	2	0	18	9	1	5	3	1024
384 (CAL67, 68)	17	10	7	0	0	21	14	5	1	1	2048
385 (NZW17)	17	13	4	0	0	19	10	3	6	0	256

TABLE 4

Number of heterologous antisera with which the indicated Bacillus anthracis and Bacillus cereus strains did not show spore agglutination

Spore Agglutinogen	LD_{50} for Mouse	*B. cereus* Antisera (12 Strains)				*B. anthracis* Antisera (10 Strains)			
		Tested*		Negative*		Tested*		Negative*	
		Strains	Sera	Strains	Sera	Strains	Sera	Strains	Sera
B. anthracis									
384S	70	12	21	0	3	8	8	3	3
385R	1×10^7	12	20	3	10	9	10	3	4
386S	330	12	21	5	13	9	10	2	2
387R	5×10^6	12	21	0	3	10	11	3	3
388S	260	12	21	12	21	10	11	9	9
389R	2×10^7	12	21	8	14	10	11	5	6
390S	200	12	21	0	3	9	9	2	2
391R	5×10^5	12	21	1	3	9	9	1	1
392S	570	12	21	12	21	10	10	8	8
393R	5×10^6	12	21	11	20	10	11	6	7
394S	1900	12	21	0	2	7	8	3	4
395R	4×10^4	12	19	1	3	7	7	3	4
399S	200	9	15	0	0	5	5	0	0
400R	7×10^7	2	2	0	0	4	4	3	3
1		6	9	1	2	6	6	2	3
417	13	12	21	12	21	10	11	8	9
410	35	12	21	3	6	10	11	6	7
379	15	12	21	12	21	10	11	8	9
380	10	12	21	2	3	9	10	4	5
381		12	21	1	5	9	10	3	3
Carbozoo	5×10^5	12	21	1	2	9	10	1	1
B. cereus									
NRS1256		11	20	2	7	10	14	10	14
NRS617		11	18	0	0	7	9	5	5
NRS847†		0				0			
NRS946†		0				0			
NRS232		11	19	0	0	10	14	4	4
NRS244		11	19	2	7	10	14	10	11
NRS249		11	21	0	0	10	14	3	4
NRS701		12	21	0	0	10	14	4	6
NRS793		11	19	0	0	10	14	0	0
NRS201		11	20	2	7	10	14	2	3
NRS201A		12	21	0	0	10	14	1	1
Ba2(1)		12	21	3	7	10	14	10	14
Ba2(10)		12	21	0	0	10	14	1	1
Ba2(16)		11	19	1	2	10	14	8	12
Ba2(19)		11	20	1	2	10	14	7	11
Ba2(20)		12	21	0	2	10	14	0	0
Ba2(22)		11	19	2	4	10	14	6	7
Ba2(23)		11	19	1	3	10	14	3	6
Ba2(24)		12	21	1	4	10	14	8	12

* The greater number of sera than strains used to produce the antisera represents duplication of antisera production with individual strains in different rabbits.

† These strains on repeated cultivation gave spore crops that spontaneously agglutinated so that agglutination titers with these strains could not be obtained. These spores did induce antibody causing agglutination of spores of other cultures.

TABLE 5

Agglutinin titers of endospores of Bacillus anthracis and Bacillus cereus tested against antiserum adsorbed with spores of a strain of the heterologous species

Antiserum Adsorbed	Strain Used for Adsorption	Results of Agglutinin Titrations on Adsorbed Serum					
		Anthrax strains tested for reduction in titer			*B. cereus* strains tested for reduction in titer		
		Total no.*	Agglutinin titer		Total no.*	Agglutinin titer	
			Reduced	Not reduced		Reduced	Not reduced
B. anthracis	*B. cereus*						
Carbozoo(8, 9)	NRS249	7	0	7	10	4	6
381(NZW53, 76)	NRS201A	6	3	3	4	3	1
395(NZW38, 42)	Ba2(22)				9	7	2
390(NZW45, 46, 48)	NRS249	8	1	7	8	6	2
400(NZW30, 58, 59, 63)	Ba2(16)	16	4	12	14	0	14
394(NZW32, 33, 34)	Ba2(20)	3	3	3	7	1	6
B. cereus	*B. anthracis*						
NRS232(D5, 6)	384	7	5	2	17	4	13
NRS1256(67A)	390	8	6	2	17	3	14

* In this total the strain used to adsorb the antiserum is not included.

Research Establishment, Porton, England, gave agglutinin reactions with spores of 13 of 16 *B. cereus* and 1 of 21 *B. anthracis* strains tested.

In spite of the cross reactions there are individual strains which in contrast to the majority of the strains do not cross react with many or any of the antisera of the heterologous species. The data, therefore, have been presented in table 4 to demonstrate this fact. *B. anthracis* spores of 5 strains, 388, 392, 393, 417, and 379, did not agglutinate with the *B. cereus* antisera. It would be a fortunate circumstance if these results were attributable to the absence of cross reacting species antigens in the endospores of these organisms. This explanation is unlikely. These same strains are also those which show the least tendency to react with antisera prepared against other strains of the homologous species (table 4). Of itself, this fact could mean that the spores of these strains in their agglutinogen content are strain specific, being lacking in antigens held in common with other strains of the parent species. Proof of this situation would be the inability of antisera prepared against spores of these strains to agglutinate spores of heterologous strains of the parent and other species. Data of this kind available for some of the *B. cereus* strains do not show this result (table 4). Thus, antiserum against strain NRS244 agglutinates spores of all of 17 *B. cereus* and 12 of 18 *B. anthracis* strains.

The capacity of a strain to induce agglutinin against a variety of strains when it, itself, is incapable of being agglutinated by antisera against these same strains may appear to be paradoxical. An explanation could be that the physical-chemical character of the surface of spores of some strains do not yield readily to agglutinating forces in spite of their reaction with agglutinin. The test for this hypothesis would be to observe for adsorption of agglutinin in the absence of agglutination. Another possible explanation is that the endospore is composed of a number of antigens, but, unlike other strains, only one or a few of these antigens appear on the spore surface. Thus, the spore would be capable of inducing synthesis of a multiplicity of agglutinins while being agglutinable by only one or a few of these agglutinins. An example of this kind has been reported (Doak and Lamanna, 1948).

It is obvious that we have not found strains inducing formation of antibody reacting only with the homologous species' spores. If species specific antigen exists, it will have to be unmasked by selectively adsorbing out nonspecies specific

antibody from antisera. Efforts in this direction, using a single strain as adsorbent, were not promising (table 5). Whether a properly selected battery of strains can be found to remove by adsorption all antibodies but species specific antibody is questionable but can be properly considered a suitable subject for future research.

A cursory examination of precipitinogens isolated by acid hydrolysis of spores revealed cross reactions by spore antisera against the two organisms. Thus, the antigenic relationships found are not limited to agglutinogens.

One school of thought maintains that *B. cereus* as a species includes *B. anthracis* as a pathogenic variety (Smith *et al.*, 1952; Brown *et al.*, 1958*a*,*b*). The present findings of antigenic relationships between the endospores of the two organisms is not in conflict with this concept but cannot be considered as final proof of the contention. A strong case for the independent identity of *B. anthracis* as a species has been made on the basis of studies of characteristics other than antigenic ones (Nordberg, 1953; Burdon, 1956; Leise *et al.*, 1959).

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SUMMARY

Rabbit antisera against endospores of *Bacillus anthracis* and *Bacillus cereus* were prepared and studied by agglutination tests. Antigens specific to the spore phase were shown to exist. The antigenic constitution of the endospores of both organisms is complex and includes both intra- and interspecies agglutinogens. It was not possible to distinguish between the endospores of the two organisms by means of agglutination tests.

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