ON THE ANTIGENIC STRUCTURE OF THE BACTERIAL SPORE

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Defalle (1902), Mellon and Anderson (1919), Howie and Cruickshank (1940), Lamanna (1940*a*, *b*), and Bekker (1944) have reported that bacterial spores are capable of inducing the formation of specific antibodies and give no evidence in agglutinin-absorption experiments of possessing antigens related to vegetative cells. In contrast to these findings is the work of Krauskopf and McCoy (1937), who, using alkali-treated spores of *Bacillus niger* to prepare antiserum, found a serological relationship of spores to vegetative cells. They concluded that spores did not possess antigenic factors not found in vegetative cells. The present series of experiments has been directed toward a more complete understanding of the spore in its antigenic relation to its mother cell and toward the reconciliation of the data of Krauskopf and McCoy with the experience of other investigators.

Cross agglutination between spores from various species, which would require a complex antigenic structure for the spore, has been denied by Bekker (1944). On the other hand, Defalle (1902) and Lamanna (1940*b*) have reported some evidence suggestive of such cross reactions, a finding which is developed further in this paper.

MATERIALS AND METHODS

The strains used were *Bacillus cereus* C3; *Bacillus subtilis*, Ford strain S8; *Bacillus vulgatus*, or Marburg strain of *Bacillus subtilis* C4; *Bacillus agri* 13; *Bacillus brevis*, Ba8 (4), a strain kindly supplied by Dr. K. L. Burdon; *Bacillus* sp. B40 (otherwise identified as *B. brevis* by Smith, Gordon, and Clark, 1946); *Bacillus sphaericus* var. *fusiformis* A20, identified by Smith, Gordon, and Clark. Unless stated otherwise, the identifications are our own.

Antisera to spores, vegetative cells and H and O antigens were produced in different rabbits by giving a series of 5 to 7 intravenous injections of 0.5 to 1 ml of the material containing 50 to 200 million cells. Vegetative cells were grown overnight fresh for each injection. H antigens were prepared by the addition of 0.2 per cent formalin to a suspension of washed vegetative cells. O antigens were prepared by boiling a suspension of washed vegetative cells for 2½ hours. Spore suspensions were obtained by seeding the cultures on asparaginate agar (Howie and Cruickshank, 1940), or beef extract agar (Lamanna, 1940*a*, *b*), and incubating for about 2 weeks at 34, 37, or 45 C depending upon the strain. Sporulation was in general more rapid and complete on asparaginate agar, although there was some variation among the species in this regard. Spores were washed thoroughly with saline and stored at 0 C. The same suspension was used for injections, agglutination trials, and absorptions.

Precipitinogens were prepared by either the formamide extraction method of Fuller (1938) or the acid extraction method (Lamanna, 1942), and sometimes by both methods. The precipitin reaction was set up as a qualitative ring test. The antigen was layered over the antiserum, and the appearance of a ring of flocculus by the end of an hour at 37 C, or overnight at 0 C, was considered positive. Proper control tubes were included.

Spore agglutinations were read with ease when the shaking method of Lamanna (1940*a*) was employed.

Technical difficulties encountered in working with this genus included spontaneous clumping of vegetative cells; *B. cereus* and *B. brevis* were the worst offenders in this respect. Though young cultures grown on nutrient agar were used frequently with success, occasionally they would unaccountably clump. The clumps were allowed to settle to the bottom of the suspension before the preparation was used.

The possibility that there may be vegetative cells, or antigenic remnants of them, in the spore suspensions is undeniable. In fact, bacillary forms could sometimes be seen microscopically. Conclusions as to spore-vegetative-cell cross reactions were, therefore, based largely on experiments in which spores were used only as agglutinative antigens, and the antisera were prepared against living fresh vegetative cell material. There is little danger of accidentally including spores in properly prepared vegetative cell vaccine.

EXPERIMENTAL RESULTS AND DISCUSSION

After the first series, or the first several series of injections, rabbit antisera to vegetative cells and to H and O antigens contained no demonstrable antibody against the homologous spore antigens (table 1). Prolonged immunization with living vegetative cell vaccine was then undertaken and found to produce an antibody that would agglutinate the spores, although in low titer. In general, the highest antispore titers accompanied the highest antivegetative-cell titers (table 2). These results suggest that there is present in the vegetative cell, in very small amounts or otherwise obscured, an antigen that also occurs in the spores.

Vegetative cells of *B. niger* were found by Krauskopf and McCoy to induce antibody against spores. To test their observation in reverse, these authors used a spore antiserum produced by injection of potassium-hydroxide-treated spores. This antiserum contained vegetative cell antibody and no specific spore antibody. They concluded from agglutination and agglutinin-absorption experiments that there were only antigenic factors common to the two stages. As a check on this work, we tried alkali treatment with spores of C3.

Potassium hydroxide in a final concentration of 5 per cent was left in contact with the spores until they would take a carbol fuchsin stain without heating. This required about 6 hours, a considerably longer time than that used by Krauskopf and McCoy. The treated spores were washed three times with 0.85 per cent saline solution and used for injection into one rabbit. Cross agglutination and reciprocal cross-agglutinin-absorption experiments were performed with

antiserum from this rabbit and with an antiserum to vegetative cells of the same strain. The results obtained are shown in figure 1. The entire block shows the titer of the unabsorbed serum. The solid fraction of the blocks indicates the titer after absorption.

TABLE 1

Agglutination tests with vegetative, O, H, and spore antigens against vegetative, O, and H antisera

	ANTISERUM																
	C3-V		C3-H			C3-O			C4-V		C4-H			C4-O			
Antigen.	C3-V	C3-S	C3-H	C3-O	C3-S	C3-H	C3-O	C3-S	C4-V	C4-S	C4-H	C4-O	C4-S	C4-H	C4-O	C4-S
Titer*...	256	0	4096	0	0	0	512	0	1024	0	256	64	0	128	4096	0

V, vegetative cells.
S, spores.
* Highest dilution of serum in which agglutination occurred.

TABLE 2

Appearance of spore antibody in vegetative cell antisera as a result of long-continued immunization

VEGETATIVE CELL ANTISERUM	NUMBER OF INJECTION SERIES AND DATE OF BLEEDING*	HOMOLOGOUS VEGETATIVE CELL TITER	HOMOLOGOUS SPORE TITER
Rabbit no. 13	1—Sept. 11, 1946	256	0
C3-V	2—Sept. 27, 1946	512	8
	3—Oct. 30, 1946	8,192	0
	4—Feb. 2, 1947	8,192	256
	5—April 27, 1947	8,192	128
	6—July 14, 1947	8,192	256
Rabbit no. 19	1—Sept. 27, 1946	256	0
C3-V	2—Oct. 30, 1946	8,192	0
	3—July 14, 1947	8,192	128
Rabbit no. 16	1—Sept. 21, 1946	16	0
S8-V	2—Oct. 30, 1946	256	4
	3—Feb. 3, 1947	1,024	64
	4—April 25, 1947	64	8
	5—July 14, 1947	512	4

* Each injection series consisted of 5 to 7 intravenous injections of antigen. The date of bleeding was approximately 10 days after the last injection of the series.

Antibody against spore and vegetative cell antigens was present in both sera, the titer being highest with the homologous antigen. Since the rabbit immunized to the vegetative antigen had received repeated series of injections of this material, the cross reaction with the spores is not surprising. One series of injections was used with the rabbit receiving alkali-treated spores, yet the vegetative cell titer was one-fourth that of the spore titer. This is worthy of attention, first,

because, according to Krauskopf and McCoy, any free vegetative cell material in the KOH-treated spore vaccine would be eliminated by the treatment, and, second, because antiserum produced in a rabbit in response to three series of injections of untreated spores showed a vegetative cell titer only $\frac{1}{64}$ that of the homologous spore titer. Evidently a vegetative cell antigen does exist in the spore, but in such a position or condition that rigorous treatment is required to expose it.

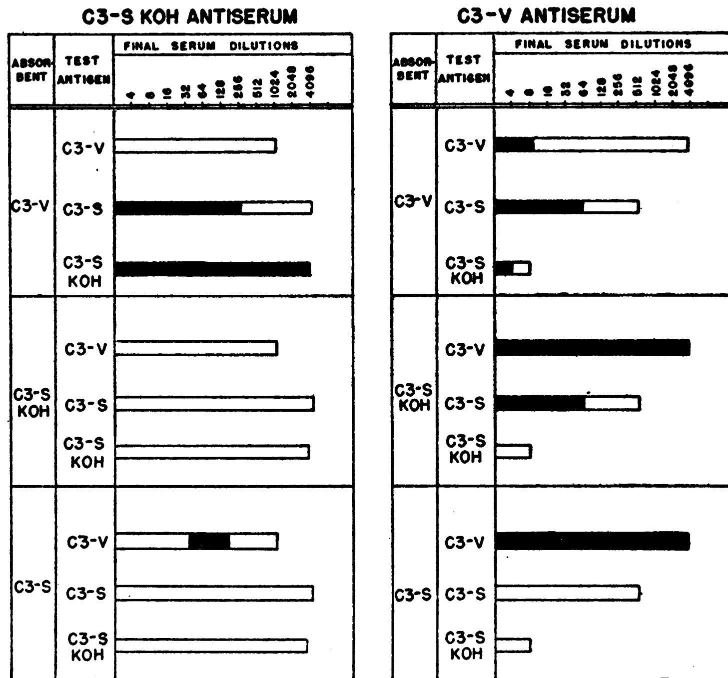

FIG. 1. AGGLUTININ ABSORPTIONS OF C3-V AND C3-S KOH ANTISERA
Entire block represents titer before agglutinin absorption. Solid portions are titer after absorption.

Absorption of the antiserum against alkali-treated spores with vegetative cells partially removed antibody against treated and untreated spores. Absorption with untreated spores removed antibody to both treated and untreated spores, and to vegetative cells, though results with the vegetative cells were not consistent on repetition. Our results differed from those of Krauskopf and McCoy chiefly in the absorptive efficiency shown by the alkali-treated spores. In the present study, the KOH-treated spores were able to induce and to absorb antibody to treated and untreated spores, and to vegetative cells, whereas treated *B. niger* spores would induce antibody against all the antigens, but only absorb antibody to themselves. Thus, one or more antigenic components, demonstrable by *in vitro* methods in the case of C3, became evident only upon animal inoculation when *B. niger* was used. Either a species difference or the longer exposure

to alkali of the spores of C3 may account for this difference between our results and those of Krauskopf and McCoy. With both organisms alkali treatment has permitted detection of an otherwise obscured antigenic relationship between spores and parent vegetative cells. In view of these results and the fact that Krauskopf and McCoy did not use untreated spores to prepare a spore antiserum, their further conclusion that specific spore antigens do not exist appears to be unwarranted.

The great complexity in the antigenic pattern of the spore became even more evident when a study of interspecies agglutination was undertaken. The results are recorded in table 3. These data reveal that spores as well as bacillary forms are capable of agglutination with antisera prepared against material from other species. That this has gone unnoticed in the work of others (Howie and Cruickshank, 1940; Bekker, 1944) is probably due to the fact that species too distantly related have been used in the attempted demonstration. For example, C3 antispore serum will not clump C4 or S8 spores, but does agglutinate *B. brevis* and B40 spores to a titer approaching the homologous system.

TABLE 3
Heterologous spore agglutination

ANTISERUM	SPORE ANTIGENS			
	C3-S	Ba8 (4)-S	13-S	A20-S
C3-S	4,096*	2,048	0	4,096
13-S	64	128	512	4
Ba8 (4)-S	2,048	2,048	16	1,024

* Highest serum dilution in which agglutination occurred.

Extensive heterologous absorptions will be necessary to determine the number and various specificities of the spore antigens. It is already clear, however, that one of the strains, C3, contains at least three surface antigens. Two of these are involved in cross absorptions with B40 (table 4). An antigen common to the C3 and B40 spores is responsible for reciprocal cross-agglutination reactions. Absorptions show that there is a second antigen in C3, not absorbable by B40 spores. A third component is indicated by agglutinations with strain 13. In this case, C3 spores are clumped by anti-13 spore serum, whereas the spores of strain 13 are not clumped by anti-C3 spore serum. This disparity in cross agglutination can be interpreted to mean that the antigen held in common by the two strains is located on the surface of the C3 spores but is absent from the surface of strain 13 spores.

Precipitating antibody is induced by spores and can be demonstrated by the use of precipitinogens prepared by either acid (Lamanna, 1942) or formamide extraction of spores. To study the relationship of the precipitinogen to the agglutinogens of spores and vegetative cells, qualitative precipitin tests were performed on spore and vegetative cell antisera before and after absorption by spores and vegetative cells (table 5). Agglutinins and precipitins were simul-

TABLE 4

Antigenic relation of C3 and B40 spores

ANTISERUM	ABSORBENT	ANTIGEN	FINAL SERUM DILUTIONS—1:										C*
			4	8	16	32	64	128	256	512	1,024	2,048	
C3-S	None	C3		+	+	+	+	+	+	+	+	+	−
		B40		+	+	+	+	+	+	+	−	−	−
	B40	C3	+	+	+	+	+	+	+	+	sl +		−
		B40	−	−	−	−	−	−	−	−	−		−
B40	None	C3	+	+	+	+	+	+	+	+	−	−	−
		B40	+	+	+	+	+	+	+	+	+	−	−
	C3	C3	−	−	−	−	−	−	−	−	−	−	−
		B40	+	+	+	+	+	+	+	+	+	−	−

* Control tube.

TABLE 5

Tests of absorption of agglutinins and precipitins from vegetative and spore antisera by agglutinogens

ANTISERUM	ABSORBENT	AGGLUTINATIONS		PRECIPITATIONS	
		Homologous vegetative titer	Homologous spore titer	Homologous vegetative antigen	Homologous spore antigen
S8-V no. 22	—	4,096	64	+ (F)	+ sl (F)
7-14-47	S8-V	0	0	0	0
	S8-S	4,096	4	+ sl*	0
S8-S no. 7	—	256	256	+ sl (F)	+ (F)
7-14-47	S8-V	0	256	0	+
	S8-S	64	0	+ sl	0
C3-V no. 13	—	4,096	0	+ (F)(A)	0 (F)(A)
10-30-46	C3-V	0	0	0	0
	C3-S	4,096	0	+	0
C3-S no. 9	—	0	256	+ (F)(A)	+ (F)(A)
11-7-46	C3-V	0	256	0	0
	C3-S	0	0	0	0
13-V	—	4,096	64	+ (A)	0 (A)
8-11-47	13-V	0	64	0	0
	13-S	4,096	0	+	0
13-S	—	128	128	0 (A)	+ (A)
7-25-47	13-V	0	128	0	+ sl
	13-S	16	16	0	0

* sl, slightly positive; (F), formamide extracts; (A), acid extracts.

taneously and specifically absorbed, that is, vegetative cells absorbed vegetative cell agglutinins and precipitins, whereas spores absorbed all and only spore antibody.

Absorption of agglutinins by precipitinogen was successful in two cases. Two S8 antispore sera, different bleedings from the same rabbit, were used. Absorption with formamide-extracted precipitinogen completely removed the antispore agglutinin titer (table 6). This procedure was not always so successful as for the case recorded in table 6. The difficulty increased as the agglutinin titer became higher, part, and sometimes all, of the agglutinin titer remaining even when further addition of precipitinogen caused no precipitate to appear. Thus in these cases after complete removal of precipitin even the presence of excess precipitinogen did not suppress or inhibit the agglutination reaction. The likelihood that the precipitinogen is a haptene fraction of an agglutinogen is suggested by the complete susceptibility of the precipitating antibody to removal by absorption by agglutinogen. That not all the agglutinogens possess the ex-

TABLE 6

Absorption of agglutinins and precipitins from spore antisera by precipitinogens

ANTISERUM	ABSORBENT	AGGLUTINATIONS		PRECIPITATION
		Homologous vegetative titer	Homologous spore titer	Homologous spore antigen (F)
S8-S	—	32	128	+
2- 3-47	S8-S (F)	64	4	0
S8-S	—		128	+
4-25-47	S8-S (F)		0	0

(F), formamide extract.

tractable carbohydrate haptene is concluded from the cases in which the precipitin absorption was incapable of seriously reducing the agglutinin titer.

The biological significance of the data presented lies in the demonstration of the antigenic complexity of the bacterial spore. Like all other living cells the spore proves to be composed of a mosaic of antigens. Some of these antigens are characteristic of the spore, whereas others are held in common with the parent vegetative cell. The fact that the spore does possess antigens not found in vegetative cells provides definite evidence of important chemical differences in the composition of complex organic molecules of spores and vegetative cells. The demonstration of carbohydrate haptene material peculiar to spores means that the carbohydrate as well as the protein composition of spores is a proper field of study in any attempt to determine the chemical basis for the unique characteristics of the spore with regard to resistance and viability. The antigenic differences and relations between spores of various species possibly indicate a fertile and relatively ignored field of study for taxonomic purposes. From the medical point of view, the distinct antigenic qualities of bacterial spores make it theoretically necessary to consider immunity against spores apart from immunity

to vegetative cells in any serious attempts to understand infection by spore-formers, and in the development of proper specific prophylactic procedures.

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CONCLUSIONS

The bacterial spore has an antigenically complex structure, a complexity extending to the cell surface. The spore possesses antigens characteristic of itself as well as others held in common with the parent vegetative cell.

An agglutinin for spores can be demonstrated in anti-vegetative-cell serum, and by the injection of alkali-treated spores to prepare antiserum a vegetative cell agglutinogen can be shown to exist in spores. The location of these common agglutinogens mostly inside the spore is suggested by two facts; first, that special procedures such as alkali treatment or extended immunization, are required to discover them, and, second, that absorption by untreated spores is specific for spore agglutinins, and absorption by living vegetative cells is specific for the vegetative cell agglutinins.

Carbohydrate type haptenes that will yield precipitates in the precipitin reaction may be isolated from spores. The location of precipitinogens on the spore surface is probable, since absorption of agglutinins will also remove precipitin.

Cross reactions between spores from different species can be demonstrated by the agglutination procedure when closely related organisms are used.

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