THE SEROLOGIC AGGLUTINATION OF BACILLUS SORDELLII AND CLOSTRIDIUM OEDEMATOIDES

IVAN C. HALL AND ALBERT L. SCOTT

Department of Bacteriology and Public Health, University of Colorado School of Medicine, Denver, Colorado

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The work of Humphreys and Meleney (1928) and of Hall, Rymer and Jungherr (1929) firmly established the identity of *Bacillus Sordellii* and *Clostridium oedematoides* on the basis of cultural and morphologic comparisons and toxin-antitoxin cross protection tests. Hall (1929) later identified two cultures of unknown anaerobic bacilli sent to him by Dr. L. R. Vawter of Reno, Nevada as *B. Sordellii* in the same manner.

In none of the preceding comparative studies, however, was the possibility of serologic identification by means of the agglutination reaction satisfactorily demonstrated. Sordelli (1923) evidently tried agglutination, for he said, "l'agglutination n'a pas permis d'etablir ni difference, ni analogie," but he gave no experimental results. Humphreys and Meleney (1928) stated that, "Agglutination reactions in this group" (the pathogenic clostridia) "are notoriously unreliable, owing, on the one hand, to the indefinite number of subgroups likely to be encountered, and on the other, to their frequent tendency to spontaneous agglutination." The work reported in this paper is a continuation of the comparative studies reported by Hall, Rymer and Jungherr in 1929, and deals with cross agglutination studies on the eight available strains of *B. Sordellii* and *C. oedematoides*.

The following cultures were investigated:

B. Sordellii 1302, separated from Sordelli's "*B. oedematis sporogenes* F9" as "solid" dissociate by J. P. Scott. Renamed by Hall and Scott (1927); pathogenic.

B. Sordellii 1303, separated from Sordelli's "*B. oedematis-sporogenes* F9" as "fluffy" dissociate by J. P. Scott. Renamed by Hall and Scott; pathogenic.

Clostridium oedematoides 1316, received from Frank Meleney, Presbyterian Hospital, New York, as "strain no. 1 from patient;" pathogenic.

Clostridium oedematoides 1317, received from Frank Meleney, Presbyterian Hospital, New York, as "strain no. 2 from catgut;" pathogenic.

Clostridium oedematoides 1318, received from Frank Meleney, Presbyterian Hospital, New York as "strain no. 3 from catgut;" pathogenic.

B. Sordellii 1322, isolated from Sordelli's "*B. oedematis-sporogenes* 82." Renamed by Hall and Scott; non-pathogenic.

B. Sordellii 2782, received from L. R. Vawter, University of Nevada, Reno, Nevada, as culture 6618 from bovine mesenteric lymph gland; pathogenic.

B. Sordellii 2783, received from L. R. Vawter, University of Nevada, Reno, Nevada, as culture 7441 from bovine liver infarct; pathogenic.

IMMUNIZATION OF ANIMALS

Our first problem was the production of potent agglutinating sera in rabbits. Due to the toxicity of the cultures, considerable difficulty was encountered. Jungherr (1927) and Hall, Rymer and Jungherr (1929) reported the same trouble, especially with intravenous injections.

Two attempts were made to immunize rabbits with small doses of whole culture without first producing antitoxic immunity; these were unsuccessful resulting in the early death of the animals, even when amounts as small as 0.01 cc. were used subcutaneously.

Attempts to use killed cultures of bacilli were unsuccessful. Cultures killed by heat have been used by Tulloch and Bauer and Meyer (1926) in the preparation of anti-tetanus agglutinating sera, and by Schoenholz and Meyer (1923) to produce anti-botulinus sera. Jungherr (1927) claims to have produced strong agglutinins for *B. Sordellii* and *C. oedematoides* by this method, although he could not demonstrate any reaction after two injections, as had Bauer and Meyer with tetanus; in his hands at least 15 injections totaling 19 cc. were necessary. We were unable to demonstrate agglutinins after a total of 45 cc. of antigen had been given.

TABLE 1

Immunization of rabbit R19 against C. oedematoides 1318

DATE	WEIGHT	DOSE	MATERIAL	ROUTE
1929	*grams*	*cc.*		
August 10	3,250	1 (1:50)	Toxin	Subcutaneous
August 17	3,250	1 (1:50)	Toxin	Subcutaneous
August 24	3,350	1 (1:40)	Toxin	Subcutaneous
August 31	3,425	1 (1:30)	Toxin	Subcutaneous
September 7	3,450	1 (1:25)	Toxin	Subcutaneous
September 11	3,500	1 (1:25)	Toxin	Subcutaneous
September 15	3,550	1 (1:25)	Toxin	Subcutaneous
September 20	3,550	1 (1:10)	Toxin	Subcutaneous
September 25	3,600	1 (1:25)	Toxin	Subcutaneous
October 10	3,625	1 (1:25)	Toxin	Subcutaneous
October 20	3,650	1 (1:25)	Toxin	Subcutaneous
November 1	3,675	1 (1:25)	Toxin	Subcutaneous
November 15	3,600	1 (1:10)	Toxin	Subcutaneous
November 30	3,675	1 (1:5)	Toxin	Subcutaneous
December 15	3,675	1 (1:5)	Toxin	Subcutaneous
1930				
January 3	3,800	1 (1:5)	Toxin	Subcutaneous
January 18	3,700	0.3	Toxin	Subcutaneous
January 24	3,700	0.5	Toxin	Subcutaneous
February 4	3,700	1	Toxin	Subcutaneous
February 8	3,750	0.4	Toxin	Subcutaneous
February 13	3,750	0.8	Toxin	Subcutaneous
February 17	3,750	1.2	Toxin	Subcutaneous
February 22	3,700	2	Toxin	Subcutaneous
March 1	3,700	3	Toxin	Subcutaneous
March 11	3,710	5	Toxin	Subcutaneous
March 17	3,725	10	Toxin	Subcutaneous
			WHOLE CULTURE AGE	
			hours	
March 25	3,675	0.1	48	Subcutaneous
March 31	3,750	0.2	48	Subcutaneous
April 5	3,750	0.4	48	Subcutaneous
April 19	3,675	0.6	48	Subcutaneous
April 26	3,600	0.8	48	Subcutaneous
April 30	3,650	1	48	Subcutaneous
May 5	3,650	2	48	Subcutaneous
May 10	3,650	5	48	Subcutaneous
May 15	3,750	5	72	Subcutaneous
May 20	3,700	5 (×2)	72	Subcutaneous
May 24	3,675	5 (×3)	48	Subcutaneous

TABLE 1—*Concluded*

DATE	WEIGHT	DOSE	WHOLE CULTURE AGE	ROUTE
1930	*grams*	*cc.*	*hours*	
May 29	3,750	5 (×3)	48	Subcutaneous
June 3	3,700	5 (×4)	72	Subcutaneous
June 15	3,600	Bled about 15 cc. from ear; 1:2,000		
June 23	3,650	5 (×4)	48	Subcutaneous
June 27	3,650	5 (×4)	72	Subcutaneous
July 1	3,700	5 (×4)	24	Subcutaneous
July 9	3,600	5 (×4)	72	Subcutaneous
July 16	3,600	5 (×4)	24	Subcutaneous
July 18	3,600	5 (×4)	48	Subcutaneous
July 25	Bled about 15 cc. from ear; 1:200			

Total material injected: Toxin, 25.38 cc.; whole culture, 195.1 cc.

Note. Numbers in parenthesis following dosage of toxin indicate dilution; those following the dosage of whole culture indicate concentration, e.g., 5 (×4) means the growth of 20 cc. of broth culture, centrifugalized and resuspended in 5 cc. of broth for injection.

TABLE 2

Summary of injections

RABBIT	TOXIN	WHOLE CULTURE	NUMBER OF INJECTIONS
	cc.	*cc.*	
261 anti 1302	29.36	33.4	32
208 anti 1303	19.76	70.5	30
274 anti 1316	25.91	192.0	41
232 anti 1317	26.06	190.5	49
R19 anti 1318	25.38	195.1	45
206 anti 1322		97.0	11

The use of formalinized antigen prepared by the method of Weinberg and Barotte (1929) also yielded negative results in our hands.

Antitoxic immunity was first produced by the injection of graded doses of filtered toxin; when the animal could tolerate 5 to 10 cc. of undiluted toxin subcutaneously, graded doses of whole culture were injected to produce agglutinin formation, but in the case of the non-pathogenic strain, 1322, it was unnecessary to start with the filtrate.

The protocol presented in table 1 is representative of the procedure used successfully with the virulent strains.

Table 2 shows the total amounts of toxin and of whole culture used in immunizing each animal and the number of injections.

TECHNIC OF AGGLUTINATION TESTS

Antigenic suspensions were prepared by filtering forty-eight-hour glucose broth cultures through loose cotton to remove clumps. Attempts were made to use centrifugalized cultures resuspended in normal saline containing 0.3 per cent tricresol, hoping that this method would avoid the formation of gas in the tubes, but such preparations failed to agglutinate in controlled tests.

For the preliminary titrations of the sera, three dilutions, namely, 1:10, 1:100, and 1:1000, were made. One cubic centimeter of each dilution was pipetted into a test tube, 100 by 13 mm., and 1 cc. of the homologous antigen added, making the final dilutions 1:20, 1:200 and 1:2000. A control tube containing 1 cc. normal saline solution and 1 cc. of antigen was also set up. Results were read after three hours in the incubator at 37°C.

In making the final tests, the same general scheme was followed as in the preliminary tests, but the dilutions of serum used were: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120; thus the final dilutions ranged from 1:20 to 1:10,240.

PRELIMINARY TITRATION OF SERA

The results of the preliminary titrations are shown in table 3. The differences in time required to produce strong agglutinins for the various strains were striking. Thus, a satisfactory titer was reached for the non-pathogenic South American strain 1322 in about six weeks. The pathogenic South American strains, 1302 and 1303, and the New York strains, 1316 and 1317, produced good sera in five to six months. The New York strain 1318 gave us unusual difficulty; rabbit after rabbit was started only to die before the immunization was complete. This finally made us extremely cautious in pushing the dosage so that the eleven

TABLE 3

Preliminary homologous agglutination titers in serums bled at different times

SERUM NUMBER	IMMUNIZATION BEGUN	DATES SERUM DRAWN						
		A July 6, 1928	B August 1, 1928	C September 10, 1928	D November 27, 1929	E April 21, 1929	F June 15, 1930	G July 25, 1930
261 anti 1302	March 7, 1928	++ 1:200	+++ 1:2,000			± 1:160		
208 anti 1303	March 17, 1928	− 1:20	+ 1:20	++ 1:2,000		± 1:20		
274 anti 1316	March 17, 1928	+ 1:200	± 1:2,000	+ 1:2,000	+++ 1:2,000	− 1:20		
232 anti 1317	March 8, 1928	+ 1:200	− 1:20	+ 1:2,000	+ 1:2,000	− 1:20		
206 anti 1322	October 5, 1928				+++ 1:2,000	± 1:80		
R19 anti 1318	August 10, 1928						+ 1:2,000	++ 1:200

Note: All sera were titrated within four days after the bleeding.

The figures in the table indicate the highest dilution in which a positive agglutination appeared and the intensity of the reaction. Complete agglutination would be indicated by ++++. No preliminary tests were made at dilutions higher than 1:2,000.

TABLE 4

Cross agglutination tests with serum 274 anti C. oedematoides 1316

Serum drawn November 27, 1928. Test made December 4, 1928.

	STRAIN	DILUTION OF SERUM										
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	Control
B. Sordellii	1302	+++	+++	+++	++	−	−	−	−	−	−	−
B. Sordellii	1303	+++	++++	+++	+++	+++	+++	+++	++	+	±	−
C. oedematoides	1316	+++	+++	+++	++	+	−	−	−	−	−	−
C. oedematoides	1317	+++	+++	+++	+++	+++	++	+	+	+	±	−
C. oedematoides	1318	+++	+++	++	++	+	+	+	+	+	±	−
B. Sordellii	1322	++++	++++	++++	+++	+++	+++	+++	++	++	+	−
B. sporogenes	10	−	−	−	−	−	−	−	−	−	−	−
B. tyrosinogenes	106	−	−	−	−	−	−	−	−	−	−	−
B. Novyi	140	−	−	−	−	−	−	−	−	−	−	−

months actually consumed were possibly even longer than necessary, but they caused a considerable delay in completing the tests.

CROSS AGGLUTINATION

Within a few days after the homologous titer of each serum was found to have reached 1:2000, the cross agglutination tests were made. The early protocols included only the three South American and the three New York strains of *B. Sordellii* described by Hall, Rymer, and Jungherr, and certain representative strains of heterologous species, *B. tyrosinogenes* 106 and *B. sporogenes* 10 which were included because of their cultural resemblance to *B. Sordellii*, and *B. Novyi* 140 which was included because of the pathologic resemblance of the lesions produced by it in animals. These strains have already been described as to origin by Hall (1922a, 1922b). The cross tests of all sera (except that prepared against 1318) upon all of these strains were completed before January 1, 1929. The Nevada strains, 2782 and 2783, were not received until February, 1929, and not identified until April; we decided to include them also in the agglutination tests at that time, but the previously prepared sera having been exhausted, it was necessary to bleed the animals again. This was done without further immunization and the agglutination content of the sera was found to be quite low; in several instances the homologous titrations even gave negative results at 1:20 (see table 3e). Notwithstanding, the Nevada strains gave positive results in every instance, and with the serum of rabbit 206 anti 1322, a definitely positive reaction at 1:5120. It is interesting to note that serum of rabbit 208 anti 1303, in spite of its low agglutinin content, was still quite antitoxic and was used successfully in the protection experiments at this time.

We also raised the question as to whether precipitins were involved in the reactions observed. Accordingly on two occasions tests were made using uninoculated glucose broth and bacteria-free filtrates of the forty-eight-hour homologous cultures as antigens. These tests were entirely negative.

The complete study involved fifteen different protocols. Table 4 illustrates one such protocol using serum rabbit 274 anti *Clostridium oedematoides* 1316.

TABLE 5

Summary of agglutination tests with anti B. Sordellii serums

ANTIGEN	SERA					
	Rabbit 261 Anti 1302	Rabbit 208 Anti 1303	Rabbit 274 Anti 1316	Rabbit 232 Anti 1317	Rabbit R19 Anti 1318	Rabbit 206 Anti 1322
B. Sordellii 1302	+ 1:2,560	++ 1:160	++ 1:160	++ 1:10,240	+ 1:5,120	++++ 1:10,240
B. Sordellii 1303	+ 1:10,240	+ 1:10,240	+ 1:5,120	+ 1:10,240	+ 1:10,240	+ 1:2,560
C. oedematoides 1316	++ 1:10,240	− 1:20	+ 1:320	+ 1:80	+ 1:40	+ 1:40
C. oedematoides 1317	++ 1:10,240	++ 1:40	+ 1:5,120	+ 1:2,560	+ 1:640	+ 1:5,120
C. oedematoides 1318	++ 1:10,240	± 1:20	+ 1:5,120	+ 1:5,120	+ 1:640	+ 1:5,120
B. Sordellii 1322	++ 1:10,240	+ 1:1,280	+ 1:10,240	+ 1:2,560	+ 1:10,240	+ 1:1,280
*B. Sordellii** 2782	+ 1:160	+ 1:80	+ 1:2,560	+ 1:640	+ 1:5,120	+ 1:5,120
*B. Sordellii** 2783	+ 1:2,560	± 1:20	+ 1:40	+ 1:320	+ 1:5,120	+ 1:5,120
B. sporogenes 10			− 1:20	+ 1:20		+ 1:20
B. tyrosinogenes 106			− 1:20	− 1:20		+ 1:20
B. Novyi 140			− 1:20	− 1:20		− 1:20
Bacteria-free filtrate†					− 1:20	− 1:20
Uninoculated dextrose broth					− 1:20	− 1:20

* These strains were tested separately with sera freshly drawn from the rabbits which had not received any immunization for several months and whose blood no longer contained strong homologous agglutinins, except in the case of rabbit R19 anti-1318. See table 3.

† Homologous cultures for serum.

Table 5 is a composite of all the titer limits obtained in the study.

DISCUSSION

The outstanding feature of table 5 is that it shows cross agglutination in all but one of the 48 tests conducted with *B. Sordellii* and *C. oedematoides*. Although the titers in some instances are somewhat low, the occurrence of cross agglutination in so high a percentage of tests confirms the serologic identity of the various strains, and suggests that agglutination tests with a potent serum, such as anti 1302, might be used to establish identity in the case of new strains which may be found. A negative result, however, could not be considered as establishing the non-identity of strains; witness the non-agglutination of strain 1316 by serum anti 1303.

Another feature is the general failure of cross agglutination with heterologous species. The positive results in low dilution in 3 out of 6 such check tests cannot be explained, but it must be noted that although weakly positive reactions did occur in low dilutions, such low titer limits were duplicated in only 3 out of the 48 tests with the homologous species, and these were in a serum of notably low titer for all heterologous strains. This circumstance, coupled with the fact that the heterologous species were agglutinated only slightly in the lowest dilution of sera which were relatively potent for the homologous species, justifies the conclusion that there is no real cross agglutination between these species.

The negative precipitin reaction is significant as showing that the animals had not become immunized to proteins in the broth. The objection might be raised here that the usual method of conducting a precipitation test is by using the serum undiluted or only slightly diluted, and making dilutions of the antigen, but it was felt that such a procedure in this case would not give results comparable to the agglutination reactions. As it was, we feel quite sure that precipitation played no part in the results observed.

It was noted that serum anti *B. Sordellii* 1302 agglutinated

all strains except the homologous strain to the highest dilution tested, while serum anti *B. Sordellii* 1303 clumped only the homologous strain to that extent. This is interesting because of the fact that the two are sibling strains, isolated from a single parent culture as "smooth" and "fluffy" colonies, although as Hall and Scott (1927) pointed out, both colonial types might be found in an old brain culture of either.

We found *C. oedematoides* 1316 to be somewhat refractory to cross agglutination, although it gave rise to a strong agglutinating serum; *B. Sordellii* 1303, on the other hand, was readily agglutinated, but produced a serum with a strong tendency toward strain specificity. Also, strain 1316 was agglutinated to a higher titer in sera against strain 1302 than in its own serum, while strains 1317 and 1318 were more susceptible to the action of serum against strain 1316 than to their own homologous sera. We cannot attempt to explain these inconsistencies, but it is possible that the observations of Weil and Felix, White, Gardner, Olitzky, Arkwright, Braun and others on multiple antigens, which led to the qualitative receptor analysis of Weil and Felix and to the work of Felix and Robertson (1928), may yield a solution of the problem.

CONCLUSIONS

1. There is cross agglutination between all strains of *B. Sordellii* and *C. oedematoides.*

2. Sera prepared against *B. Sordellii* or *C. oedematoides* do not cross agglutinate *B. sporogenes, B. tyrosinogenes* or *B. Novyi.*

3. *B. Sordellii* and *C. oedematoides* are therefore distinct serologically from other species of obligately anaerobic bacilli.

Since the completion of our work Vawter and Records have reported in connection with their "Serologic Study of Sixteen Strains of Bacillus Hemolyticus" in the *Journal of Infectious Diseases* for July, 1931, that successful agglutinating serums were prepared by them against three strains of *B. Sordellii,* but these serums failed to agglutinate *B. hemolyticus,* also that there was no cross agglutinating of *B. Sordellii* by serums prepared with *B. hemolyticus.*

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