Fluorescent-Antibody Reagents for the Identification of *Clostridium botulinum*

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Fluorescent-antibody reagents were prepared against vegetative cells of representative strains of each physiological group and toxin type of *Clostridium botulinum* known to have caused botulism in humans. A fluorescent-antibody reagent was also prepared for *C. botulinum* type G, which has been isolated from autopsy specimens but which has not clearly been implicated in botulism. These fluorescent-antibody reagents were evaluated against 200 strains of *C. botulinum* and 64 strains of other clostridia. Each reagent reacted with at least a 2+ intensity with all of the strains in its same toxin type and physiological group. Ninety-seven percent of the strains gave at least a 3+ reaction with the homologous group or toxin type reagent. Some cross-reactions occurred with reagents against different toxin type strains within a physiological group; there was less cross-reaction between physiological groups and very little reactivity of *C. botulinum* reagents with nontoxicogenic organisms. Absorption of cross-reacting antibodies was not successful. Certain reagents could be used for presumptive laboratory identification of *C. botulinum* strains causing botulism, especially in infants. The type G reagent provided a good means of identifying *C. botulinum* type G, which lacks the lipase marker and whose toxigenicity may be more difficult to demonstrate in mixed cultures. There was a serological relationship between *C. botulinum* type G and some strains of *Clostridium subterminale*. This relationship provided evidence of differences between strains of *C. botulinum* type G isolated in two different countries.

Clostridium botulinum, although designated as a single species, actually comprises a collection of four dissimilar groups of clostridia which possess in common the ability to produce potent neurotoxins which have similar, if not identical, pharmacological modes of action. Smith (10) lists the four groups of *C. botulinum* as I, proteolytic strains of toxin types A, B, and F; II, non-proteolytic strains of toxin types B, E, and F; III, toxin types C and D; and IV, toxin type G (see Table 1). Human botulism is caused primarily, if not exclusively, by organisms of groups I and II. There have been no adequately confirmed human cases caused by groups III or IV. All cases of infant botulism confirmed thus far have been caused by group I organisms, and with one exception (7), the toxin types have always been A or B.

Although no cases of type G botulism, either human or animal, have been documented, Sonabend et al. have reported the isolation of *C. botulinum* type G from autopsies of cases of unexplained death in Switzerland (12). These findings are similar to the finding of type A and B strains in autopsies of a number of cases of sudden infant death syndrome (1) and raise the

question of whether the organisms played any role in those deaths. *C. botulinum* type G is difficult to recognize in mixed cultures because of its lack of lipase and low toxigenicity.

The purpose of our study was to evaluate whether fluorescent-antibody (FA) reagents could be reliably used to detect and identify *C. botulinum* in fecal specimens and enrichment cultures. If reliable, the use of FA techniques for presumptive diagnosis of botulism would be especially useful at the local level for diagnosing the disease in infants in situations in which mice for toxicity and neutralization tests or the expertise for performing and interpreting those tests may not be available.

We prepared FA reagents against a representative strain of each toxin type within each of the two groups of *C. botulinum* known to cause human botulism. We then evaluated them against a large number of strains of *C. botulinum* and other clostridia to determine whether they could be used reliably for diagnostic purposes. Two additional reagents were made, one against *Clostridium sporogenes* and one against *C. botulinum* type G. *C. sporogenes* resembles the group I *C. botulinum* strains except that it lacks

TABLE 1. Groups and strains of *C. botulinum* and related clostridia used for evaluation of *C. botulinum* FA reagents^a

Group	Distinctive characteristic	Toxin types	No. of strains	Related organisms ^b	No. of strains
I	Proteolytic	A	89		
		B	46	<i>C. sporogenes</i>	15
		F	6	<i>Clostridium</i> sp. (nontoxigenic isolates from previously toxic <i>C. botulinum</i> cultures)	4
II	Non-proteolytic	E	11		
		B	8	<i>Clostridium</i> sp., non-toxicogenic	9
		F	6		
III	Involved in avian and animal botulism	C	19	<i>C. novyi</i>	10
		D	8		
IV	Proteolytic, asaccharolytic, lipase negative, not implicated in botulism	G	7	<i>C. subterminale</i>	8
				<i>C. hastiforme</i>	1

^a In addition, 17 other unrelated clostridia were used: 5 strains of *C. perfringens*, 2 strains of *C. histolyticum*, and 1 strain each of *C. difficile*, *C. paraputrificum*, *C. tertium*, *C. bifermentans*, *C. sordellii*, *C. chauvoei*, *C. tetani*, *C. innocuum*, *C. cadaveris*, and *C. septicum*.

^b Organisms which have similar, if not identical, cultural and physiological characteristics but which do not produce a botulin neurotoxin.

toxigenicity. The type G reagent was prepared, although type G has not yet been found in the United States but has been isolated from human specimens in Switzerland (12).

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MATERIALS AND METHODS

Bacterial strains. All strains of clostridia used in this study came from the Centers for Disease Control Botulism Laboratory culture collection (Atlanta, Ga.). These strains include 200 strains of *C. botulinum* and 64 strains of other clostridia as listed in Table 1. The type G strains included two strains isolated in Argentina (5; A. S. Ciccarelli, A. M. Giulietti, and D. F. Gimenez, Primer Congreso y IV Jornadas Argentinas Microbiología, abstr. no. 21, 1976) and five strains isolated in Switzerland (12). All strains were identified by the Centers for Disease Control Anaerobe Section by methods described elsewhere (3). For this study, they were rechecked for purity, toxigenicity, and toxin type.

Antigen preparation. The following bacterial strains (antigen strains) were completely retested for their biochemical, physiological, and toxigenic characteristics: MD-00a10, *C. botulinum* type A; MD-000b2, *C. botulinum* type B; BL-00706, *C. botulinum* type B (non-proteolytic); BL-00211, *C. botulinum* type E (Dolman VH strain); BL-02821, *C. botulinum* type F; RH-00084, *C. botulinum* type F (non-proteolytic); BL-00714, *C. botulinum* type G; and BL02285, *C. sporogenes*. The type A and B (proteolytic) antigen strains were isolates from infants with botulism, and the type G was the second strain isolated in Argentina. The antigen strains were cultured for short periods before

harvesting to ensure the maximum yield of vegetative cells and a minimum of spores, as recommended by Midura et al. (9). Transfers of the organism were made over a period of 3 days at 4- to 12-h intervals in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.; catalog no. 12191) or Lombard-Dowell broth (4). The cultures were then centrifuged, and the sedimented cells were washed five times with saline containing 0.4% Formalin and then were suspended in formalinized saline at a density comparable to a McFarland standard no. 3. The cell suspensions were checked for sterility by inoculating 1 ml of the suspension into thioglycolate broth medium. Toxicity was checked by mouse inoculation.

Rabbit immunization. Preimmune blood was drawn by heart puncture from New Zealand rabbits weighing approximately 2.7 kg (40-ml syringe, 20-gauge needle), and the serum was separated and stored at -20°C. After 1 week, the rabbits were immunized intravenously (ear vein) with six injections at 3- to 4-day intervals with increasing amounts of the antigen (1, 2, 3, 4, 5, and 5 ml). One week after the sixth injection, the rabbits were bled by heart puncture. They were bled two or three times, and then a booster of 5 ml of antigen was given to each of the surviving rabbits. After 8 days, the rabbits were exsanguinated. The harvested serum was separated and stored at -20°C.

Serum titer determination. The antibody titer of each serum was determined against its immunizing strain by tube agglutination studies (8). Preimmune serum was used as a negative control. Each titer was recorded as the reciprocal of the highest serum dilution which caused clumping which, after centrifuging, did not break up when the base of the tube was tapped sharply.

Fractionation and conjugation of antisera. Preimmune serum (a pool from all the rabbits immunized) and the immune sera were fractionated by precipita-

tion with 50% saturated ammonium sulfate and were purified by passage through a DEAE-Sephadex A-50 column. The gamma globulin fractions were labeled with fluorescein isothiocyanate as described by Jones et al. (6). The staining titers of the FA conjugates were determined by testing twofold dilutions (1:2 to 1:128) with the immunizing strains. The titer is defined as the highest dilution giving a 4+ reaction with the immunizing strain. For use, the serum was diluted 1 dilution less than the staining titer (the use dilution). The conjugates were mixed with an equal volume of normal rabbit serum and stored in 2-ml portions at -20°C .

Evaluation of FA reagents. The FA reagents were tested against the 200 strains of *C. botulinum* (toxin types A through G), 15 strains of *C. sporogenes*, 13 nontoxigenic strains resembling *C. botulinum*, and 36 strains of other *Clostridium* species (16 species) listed in Table 1. The organisms were grown in NIH thioglycolate broth (Difco Laboratories, Detroit, Mich., code no. 0257) or on blood agar plates. Smears of the 24- to 48-h cultures were prepared on alcohol-cleaned glass slides, air dried, fixed with Kirkpatrick fixative (6), overlaid with the FA conjugates at the use dilution, and incubated for 30 min in a moist chamber at room temperature. The slides were washed with two changes of phosphate-buffered saline and one change of distilled water and were air dried. A drop of mounting fluid (90% glycerol, 10% 0.5 M sodium bicarbonate buffer, pH 9.0 [6]) was placed on each smear, and a cover slip was placed on each slide.

The smears were read on a fluorescence microscope (Leitz Dialux 20; Leitz/Opto-Metric, Rockleigh, N.J.; vertical illumination), and the intensity of fluorescence was estimated as follows: 4+, maximal fluorescence, brilliant yellow-green; 3+, less brilliant yellow-green fluorescence with clear-cut cell outlines and sharply defined nonstaining center of cell; 2+, definite fluorescence but less brilliant, less clear-cut cell outline, and nonstaining center area fuzzy; 1+, subdued cell wall fluorescence with the cell center indistinguishable from the cell wall (6).

Absorption studies. Absorption studies were done by a procedure used by Solomon et al. (11). The *C. botulinum* type A conjugate was absorbed with a strain of *C. sporogenes*. The *C. botulinum* type B (proteolytic) conjugate was absorbed with a strain of *Clostridium histolyticum*, and the *C. botulinum* type G conjugate was absorbed with a strain of *Clostridium subterminale*.

RESULTS

The agglutination titers of the rabbit sera against the immunizing strains and the FA titers and the fluorescein-to-protein ratios of the FA conjugates prepared from them are listed in Table 2. The reactivity of the group I (types A, B, and F), group II (types B, E, and F), group IV (type G), and *C. sporogenes* FA reagents with the homologous and heterologous groups of clostridial strains are listed in Table 3.

Each of the seven *C. botulinum* FA reagents reacted with at least a 2+ intensity with all of the strains of the same toxin type within the physiological group of the immunizing strain. In fact, except for five strains of type A and one strain of

group I type B, the reactions with the homologous reagents were 3+ or better. The *C. sporogenes* reagent reacted with only 7 of the 15 strains of *C. sporogenes* at an intensity of 2+ or greater. There was considerable cross-reactivity within the physiological groups I and II. Note especially the cross-reactions between toxin types B and F in group II. The type A reagent reacted with three nontoxigenic organisms (one resembling type E and two strains of *C. sporogenes*), and the B (group I) reagent reacted with four (two resembling type E, and one strain of *C. sporogenes*, and one of *C. histolyticum*). The type G reagent reacted with two strains of *C. subterminale*.

Absorption studies with cross-reacting species. Attempts to improve the specificity of the types A, B (group I), and G reagents by absorption with cross-reactive species gave results which are summarized in Table 4. Absorption of the type A reagent with a strain of *C. sporogenes* removed the cross-reacting antibodies and did not lower the FA titer for the immunizing strain but did destroy its usefulness for detecting *C. botulinum* type A. The attempted absorption of the B reagent with *C. histolyticum* failed to remove the cross-reacting antibodies. The absorption of the type G reagent with *C. subterminale* removed the cross-reacting antibodies and rendered the reagent incapable of detecting the five strains of *C. botulinum* type G previously isolated in Switzerland (12), but it was still potent with regard to the immunizing strain as

TABLE 2. Agglutination titers of immune rabbit sera with the immunizing strains and FA titers and fluorescein-to-protein ratios of the corresponding conjugate reagents

Reagent	Serum agglutination titer (tube)	Conjugate	
		FA titer	F/P ratio ^a
Group I			
A	64	64	21/1
B	64	64	17/1
F	128	128	19/1
Group II			
B	128	64	23/1
E	64	16	25/1
F	128	8	18/1
Group IV			
G	32	32	16/1
<i>C. sporogenes</i>	64	64	27/1

^a F/P ratio = (micrograms of fluorescein isothiocyanate per milliliter)/(milligrams of protein per milliliter).

TABLE 3. Percentage of clostridial strains reacting with FA reagents prepared against *C. botulinum* and *C. sporogenes* vegetative cells

Organism	No. of strains	% Reacting with FA reagents ^a :							
		<i>C. botulinum</i> group I type:			<i>C. botulinum</i> group II type:			<i>C. botulinum</i> group IV, type G	<i>C. sporogenes</i>
		A	B	F	B	E	F		
Group I									
<i>C. botulinum</i> type A	89	100	22	7	4	21	8	9	0
<i>C. botulinum</i> type B	46	30	100	2	4	17	6	6	0
<i>C. botulinum</i> type F	6	16	33	100	0	33	16	0	0
Group II									
<i>C. botulinum</i> type B	8	25	25	25	100	75	100	12	25
<i>C. botulinum</i> type E	11	9	18	0	18	100	27	0	9
<i>C. botulinum</i> type F	6	0	0	0	83	0	100	0	0
Group III									
<i>C. botulinum</i> type C	19	0	0	0	0	0	0	0	0
<i>C. botulinum</i> type D	8	13	0	0	0	0	25	0	0
Group IV									
<i>C. botulinum</i> type G	7	0	0	0	0	0	28	100	0
Nontoxigenic, derived from <i>C. botulinum</i> cultures									
Type A	2	0	0	0	50	0	0	0	0
Type B (group I)	2	0	0	0	0	0	0	0	0
Type B (group II)	1	0	0	0	0	0	0	0	0
Type E	3	0	0	0	0	0	0	0	0
Nontoxigenic when isolated, resembling									
<i>C. botulinum</i> type E	5	20	40	0	40	20	0	0	40
<i>C. sporogenes</i>	15	13	7	0	0	7	0	0	47
<i>C. subterminale</i>	8	0	0	0	0	0	0	25	0
<i>C. perfringens</i>	5	0	0	0	0	0	60	0	0
<i>C. histolyticum</i>	2	0	50	0	0	0	0	0	0
Other clostridia	21	0	0	0	0	0	0	0	0

^a Reactions of 2+ or greater are recorded as positive.

well as the other (original) isolate from Argentina (5).

DISCUSSION

Although a degree of specificity was attained with each reagent produced against a representative organism of a group and toxin type, the specificity was not absolute. Considering the group I reagents, a 2+ intensity of staining should be considered a positive reaction to ensure that none of the type A or B organisms are missed. This increases the number of cross-reacting organisms that are included, especially those in groups I and II. Since the group I reagents react with a few nontoxigenic organisms, the mouse tests are subsequently required to confirm toxigenicity and to identify positively the toxin type by specific neutralization. If a culture or specimen is FA negative with the group I reagents, proteolytic *C. botulinum* is either absent or present in numbers too low to be

observed. If a cross-reaction occurs between the reagent and a strain of *C. botulinum* of a different toxin type, even if it belongs to a different physiological group, the finding of *C. botulinum* would appear to be just as valid for confirmatory evidence of botulism. Thus, the use of a divalent AB (group I) reagent could be used for testing for the presence of *C. botulinum* in stool specimens and stool enrichment cultures from infants with suspected botulism. FA-positive cultures would ultimately have to be confirmed by mouse tests.

One hundred percent of the group II strains reacted at the 3+ level with their homologous group and toxin type reagents, although there were a smaller number of strains of group II organisms for the evaluation. The cross-reaction of the type F (group II) reagent with three of five strains of *Clostridium perfringens* makes its usefulness questionable because *C. perfringens* is likely to be found, sometimes in high numbers,

TABLE 4. Absorption of *C. botulinum* FA reagents types A, B (group I), and G

Reagent	Before or after absorption	Cross-reaction with absorbing strain (intensity):			Titer with immunizing strains of <i>C. botulinum</i> :			% of homologous group and toxin type <i>C. botulinum</i> strains with reaction of 2+ or better		
		<i>C. sporogenes</i> 218	<i>C. histolyticum</i> 1942	<i>C. subterminale</i> 4422A	Type A	Type B	Type G	Type A (27 strains)	Type B (31 strains)	Type G (7 strains)
A	Before	2+			64			100		
	After	—			64			22		
B (group I)	Before		4+			64			100	
	After		4+			64			97	
G	Before			3+			32			100
	After			—			32			28

in specimens which may be examined for botulism. The type E reagent cross-reacts with 27 group I strains but cross-reacts with very few nontoxigenic organisms.

All reported botulism from group II strains in the United States has been foodborne and almost entirely limited to type E (2; Centers for Disease Control, unpublished data). The type E reagent prepared for these studies could be used in combination with the group I type A and B reagents for investigation of foodborne botulism with the likelihood of only a few false-positives.

The *C. sporogenes* FA reagent failed to react with any of the 141 group I strains of *C. botulinum* and reacted with only half of the *C. sporogenes* strains. Although *C. sporogenes* differs from *C. botulinum* only in its lack of toxin production, the *C. sporogenes* FA reagent did not react with any of the strains which may represent *C. botulinum* that had lost toxigenicity.

It would be advantageous to distinguish group I *C. botulinum* from *C. sporogenes* without performing toxicity tests. On the basis of our results, we could suggest the following. (i) Failure to react with the group I type A, B, and F reagents would indicate that the organisms are probably nontoxigenic. (ii) A positive reaction with one or more of the group I reagents would indicate a high probability that the organism is toxigenic, but confirmation by a toxin test is still necessary.

The type G (group IV) reagent successfully identified all seven strains of *C. botulinum* type G in this study but cross-reacted with two strains of *C. subterminale*, an organism which resembles *C. botulinum* type G in all physiological characteristics except for toxigenicity. *C. subterminale* is related to group IV; the relationship is similar to that of *C. sporogenes* to group I. Past isolates of *C. botulinum* type G not shown to be toxigenic may have been misidentified as *C. subterminale*. Failure to establish

toxigenicity may have been the following: failure to perform mouse tests, low culture toxicity, too short an incubation period, or inadequate media. Sometimes, type G cultures require trypsinization for toxin demonstration. Absorption with *C. subterminale* removed activity for the five Swiss strains of *C. botulinum* type G but not for the two Argentine strains. The isolates from the two locations were clearly different serologically.

In conclusion, the results of this study indicate that FA reagents prepared against vegetative cells of *C. botulinum* can be used to aid in the diagnosis of botulism. In particular, the use of group I reagents can provide a rather high probability of confirming the presence of an organism in stool specimens from infants with botulism. An evaluation of the use of these reagents for examining clinical specimens was carried out, and the results of that study will be reported later. The type G reagent shows promise as a means of identifying *C. botulinum* type G in clinical specimens.

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