Antigenic Relationships Among the Proteolytic and Nonproteolytic Strains of Clostridium botulinum

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Relationships of the somatic antigens among Clostridium botulinum strains have been investigated by tube agglutination and agglutinin absorption tests. Results revealed a relationship by which strains of C. botulinum are grouped by their proteolytic capacity rather than by the type of specific toxin produced. Thus, C. botulinum type E and its nontoxicogenic variants, which are nonproteolytic, share common somatic antigens with the nonproteolytic strains of types B and F. Absorption of antiserum of a strain of any one type with antigen of any of the others removes the antibody to all three types. In the same manner, C. botulinum type A shares somatic antigens with the proteolytic strains of types B and F, and absorption of any one antiserum with an antigen of either of the other two types removes the antibody to all three types. Partial cross-agglutination of C. sporogenes, C. tetani, and C. histolyticum with the somatic antisera of the proteolytic group was also observed.

The classification of strains of Clostridium botulinum into toxigenic types A through F is based on the antigenic specificity of the toxin produced while their biochemical activity separates them into proteolytic and nonproteolytic strains regardless of the type. In recent years nonproteolytic strains of C. botulinum type B have been isolated in North America (3, 5), and proteolytic and nonproteolytic strains of C. botulinum type F have been found in various geographical areas (2, 4, 8, 13, 17).

The fluorescent-antibody technique has shown that proteolytic strains of C. botulinum types A, B, and F share common somatic antigens (16). However, the existence of both proteolytic and nonproteolytic strains within the same toxigenic type raises the question of the relationship of their somatic antigens. Whereas nonproteolytic strains of types B, E, and F have been shown to be similar in their physiological characteristics (5, 6), very limited information on the antigenic relationship among them has been reported (H. M. Solomon et al., Bacteriol. Proc., p. 19, 1969). This investigation was undertaken to determine the relationship between the somatic antigens of proteolytic and nonproteolytic strains of a given toxigenic type, to determine whether an antigenic relationship exists among the nonproteolytic strains of types B, E, and F, and to confirm the relationship among the somatic antigens of the proteolytic strains of types A, B, and F.

MATERIALS AND METHODS

Strains. The strains investigated are identified in Table 1. Many of them have been studied in our previous work (11, 15). Other clostridial species were included in order to evaluate the specificity of the somatic antisera. Methods for the propagation of the strains used in this study have been previously described (9).

Immunizing antigens. The desired strains were grown for 16 to 18 hr in Trypticase (BBL)-peptone-glucose (TPG) broth of Schmidt et al. (14) containing 0.1% rather than 0.2% sodium thioglycolate. The proteolytic strains were incubated at 35 C and the nonproteolytic strains at 26 C. Occasionally the incubation period was extended in order to improve cell yields. All cultures were examined microscopically to insure that only vegetative cells were present. The cultures were steamed at 100 C for 1 hr, and the cells were then separated by centrifugation and washed three times with physiological saline. After the optical density (OD) of the suspensions was adjusted with physiological saline to approximately 0.5 on a Coleman colorimeter, model 8, at a wavelength of 655 nm, Formalin was added as a preservative to a final concentration of 0.2%. At this OD the suspensions contained 10^6 to 10^7 cells per ml as determined in TPG broth by the serial tube dilution technique on parallel viable cultures. The antigens were tested for toxicity and sterility and stored under refrigeration until used.

Preparation of antisera. Hyperimmune antisera for
TABLE 1. Identification of strains of Clostridium

| Strain designation | Supplied by |
|--------------------|-------------|
| *Clostridium botulinum* | |
| **Proteolytic strains** | |
| Type A | (See reference 15) |
| 62A | National Canners Association, Washington, D.C. |
| 73A | Food and Drug Administration |
| 426A | |
| Type B | (See reference 15) |
| 3A-B, 32B, 115B, 213B | National Canners Association, Washington, D.C. |
| 169B | |
| Type F | |
| Langeland-F | L. V. Holdeman, Virginia Polytechnic Inst., Blacksburg, Va. |
| 8G-F, 4VII-F | N. W. Walls, Georgia Institute of Technology, Atlanta, Ga. |
| **Nonproteolytic strains** | |
| Type B | |
| 2B, DB2, 17B | M. W. Eklund, Bureau of Commercial Fisheries, Seattle, Wash. |
| Type E | (See reference 15) |
| Toxigenic strains | |
| Beluga, VH, Me-manbetsu, 1304, D8, 070, Kalamazoo | |
| Nontoxigenic variants | (See reference 15) |
| S5, GB3, 28-2, 810, 833, 066BNT, 38-1 | |
| Nontoxigenic atypical | (See reference 15) |
| PM15, S9, 42-2 | |
| Type F | |
| 70F, 83F, 190F | M. W. Eklund (see above) |
| 202F, 205F | J. M. Craig, Oregon State University, Corvallis, Oregon |
| 610F | |
| Type C (526C) | (See reference 15) |
| Type D (ATCC) | American Type Culture Collection |
| Other *Clostridium* species | (See reference 15) |
| *C. perfringens* | J. Evans, Walter Reed Institute of Research, Washington, D.C. |
| *C. sporogenes* | |
| *C. bifermentans* | |
| *C. bifermentans* KA91 | |
| *C. sordelli* (CN1734) | |
| *C. tetani* | |
| *C. histolyticum* | |
| *Clostridium* sp. KA89, KA94 | (See reference 15) |

Agglutination tests were produced in New Zealand white rabbits. The rabbits were injected intravenously with 1.0 ml of antigen every 3 to 4 days for a total of six injections. After 14 days rest, they were given another 1.5-ml injection and were bled from the ear 10 days later. Four more bleedings were obtained with the same booster schedule. The antisera tities of all bleedings were approximately the same. Antiserum was stored frozen without preservative.

**Agglutinating antigens.** Antigens for somatic agglutinations were prepared from 16- to 18-hr TPG broth cultures grown at either 26 C or 35 C with occasional extension of the incubation period to improve cell yields. The cultures were steamed at 100 C for 1 hr, and the cells were separated by centrifugation and resuspended in physiological saline to the same OD as that of the immunizing antigens. Phenol was added to the suspensions to a final concentration of 0.2%.

**Agglutination tests.** Agglutination tests were performed in 13- by 100-mm tubes by mixing 0.1-ml portions of the antigen with an equal amount of twofold dilutions of antiserum. The tubes were incubated for 2 hr in a 50 C water bath. Physiological saline containing 0.2% phenol was used to dilute the antiserum. After incubation 0.5 ml of the same diluent was added, and the results were read immediately with a 3.5 X hand lens using a bright light against a black background. Gently flicking the tubes facilitated the determination of end points. Titer were read as the reciprocal of the highest dilution giving definite agglutination.

**Agglutinin absorption.** Cells for the absorption of antibody were grown for 16 to 18 hr in TPG at either 26 or 35 C, steamed at 100 C for 1 hr, and packed by centrifugation. Portions (2 ml) of the undiluted antiserum were mixed with 1 to 2 ml of packed cells and incubated at 50 C for 2 hr with occasional shaking. After the cells were removed by centrifugation, the antiserum was tested against the absorbing strain for antibody content. If agglutinins to the absorbing strain remained, the absorption was repeated. In all cases, an additional portion of the same antiserum was incubated as a control along with the one being absorbed.

**RESULTS**

Agglutination of proteolytic and nonproteolytic strains. High titers of somatic antibody were produced by all strains. Table 2 summarizes the agglutination of somatic antigens by somatic antiserum of the proteolytic and nonproteolytic strains of *C. botulinum*. All antiserum to the proteolytic strains of types A, B, and F reacted with the proteolytic strains of the homologous type and equally well with all the proteolytic strains of the other two types. However, none of these antiserum reacted with any of the nonproteolytic strains. Similarly, the nonproteolytic strains of types B, E, and F completely cross-agglutinated, but the antisera against the nonproteolytic strains did not react with the proteolytic strains. Thus, although there was complete cross-agglutination...
TABLE 2. Somatic agglutinations of proteolytic and nonproteolytic strains of Clostridium botulinum

| Antigens | Proteolytic strains | Nonproteolytic strains |
|----------|---------------------|------------------------|
|          | A (1)       | B (2)       | F (2)       | B (2)  | E (7)  | F (3)  |
| Type A (3) | 2560   | 2560   | 1280   |       |       |       |
| Type B (5) | 2560   | 2560   | 1280   |       |       |       |
| Type F (3) | 2560   | 2560   | 1280   |       |       |       |
| Type E    |           |           |         |       |       |       |
| Toxigenic strains (7) |       |       |       |       |       |       |
| Nontoxigenic variants (7) |       |       |       |       |       |       |
| Boticin + (5) |       |       |       |       |       |       |
| Boticin - (2) |       |       |       |       |       |       |
| Nontoxigenic atypical (3) |       |       |       |       |       |       |
| Type F (6) | 2560   | 2560   | 2560   |       |       |       |
| Type C    |           |           |         |       |       |       |
| Type D    |           |           |         |       |       |       |

* Values shown are mean titers with a range of one twofold dilution above and below. Numbers in parentheses indicate the number of strains tested. Where no values appear, agglutination did not occur at 1:20.

† Strains: 62A; 115B, 169B; Langeland-F and 8G-F.

‡ Strains: 2B, 17B; type E, toxigenic: 070, VH, 1304, nontoxigenic variants: S5, 28-2, 066BNT, and nontoxigenic atypical: S9; 83F, 202F, 610F.

TABLE 3. Agglutinin titers of proteolytic somatic antisera absorbed with proteolytic somatic antigens

| Antiserum | Absorbing strain | Antigen* |
|-----------|------------------|----------|
|           |                  | A (3)    | B (5)   | F (3)   |
| 62A       | None             | 2560     | 2560   | 2560   |
|           | 62A              |          |        |        |
|           | 169B             |          |        |        |
|           | Langeland-F      |          |        |        |
| 169B      | None             | 2560     | 2560   | 2560   |
|           | 62A              |          |        |        |
|           | 169B             |          |        |        |
|           | Langeland-F      |          |        |        |
| Langeland-F | None         | 1280     | 1280   | 1280   |
|           | 62A              |          |        |        |
|           | 169B             |          |        |        |
|           | Langeland-F      |          |        |        |

* Numbers in parentheses indicate the number of strains tested. Where no values appear, agglutination did not occur at 1:20.

Both the type E antisera and the antisera to the nonproteolytic strains of types B and F agglutinated to full titer the nontoxigenic variants of type E, including the nontoxigenic atypical strains. The type C and D strains, although nonproteolytic, were not agglutinated by the antisera to the nonproteolytic strains of types B, E, and F nor by any of the antisera to the other C. botulinum strains used in this investigation.

Antigenic similarity of strains. The similarity of the somatic antigens of the proteolytic strains is shown by the fact that removal of the antibodies of any one strain by absorption removed antibodies to all the others (Table 3). Thus, when the somatic antiserum for the proteolytic type A strain (62A) was absorbed with either the homologous antigen, the proteolytic type B strain (169B), or the proteolytic type F strain (Langeland-F), antibodies to all proteolytic strains against which it was tested were removed. Similar results were obtained when the somatic antisera to the 169B strain or the Langeland-F strain were absorbed with the same antigens.

Comparable data are presented in Table 4 for the somatic antisera of the nonproteolytic strains of types B, E, and F. As in the case of the proteolytic strains, absorption of the antibodies for any one strain removed antibodies to all the others. Thus, when the somatic antiserum to
the nonproteolytic type B strain (17B) was absorbed with either the homologous antigen, the VH strain of type E, or the nonproteolytic type F strain (202F), antibodies to all nonproteolytic strains of the three types were removed. Again, when the somatic antiserum to the VH strain or the 202 F was absorbed with the same antigens, similar results were obtained.

Other Clostridium species. Cross-agglutination

Table 4. Agglutinin titers of nonproteolytic somatic antiserum absorbed with nonproteolytic somatic antigens

| Antiserum | Absorbing strain | Antigensa |
|-----------|------------------|-----------|
|           |                  | B (3)     |
| 17B       | None             | 2560      |
|           | 17B VH-E 202F    | 2560      |
| VH-E      | None             | 2560      |
|           | 17B VH-E 202F    | 2560      |
| 202F      | None             | 2560      |
|           | 17B VH-E 202F    | 2560      |

a Numbers in parentheses indicate the number of strains tested. Where no values appear, agglutination did not occur at 1:20.

b Of the type E strains, seven were toxigenic and six were nontoxigenic variants.

Table 5. Somatic cross-agglutination of proteolytic and nonproteolytic strains of Clostridium botulinum with other Clostridium species

| Antigens                          | Antiserum againstb |
|-----------------------------------|---------------------|
|                                   | Proteolytic strains | Nonproteolytic strains | C. bifermensc | C. sporogensch |
|                                   | A  | B  | F  |     |       |       |
| Clostridium perfringens . . . . . .|    |    |    |     |       |       |
| C. bifermentans  . . . . . . . . .| 160| 160| 160| 1280|       |       |
| C. bifermentans KA91 . . . . . .  |    |    |    | 640 |       |       |
| C. sordelli . . . . . . . . . . . |    |    |    | 640 |       |       |
| C. sporogenes . . . . . . . . .   | 320| 160| 160| 1280|       |       |
| C. tetani . . . . . . . . . . .  |    |    |    | 160 |       |       |
| C. histolyticum . . . . . . . . .| 40 | 40 | 40 | 40  |       |       |
| Clostridium sp. KA89 . . . . . . |    |    |    |     |       |       |
| Clostridium sp. KA94 . . . . . . |    |    |    |     |       |       |

a The same kinds and numbers of antisera were tested as in Table 2. Where no values appear, agglutination did not occur at 1:20.

b C. bifermentans antiserum did not agglutinate any of the antigens listed in Table 2.

c C. sporogenes antiserum agglutinated all the proteolytic strains of A, B, F to a titer of 1:160.
produce the same type of toxin do not share somatic antigens.

The antigenic similarity of the nonproteolytic botulinal organisms is consistent with other characteristics of these organisms. Eklund et al. (5, 6) have shown that the nonproteolytic type B and F strains are similar to type E in their ability to grow and produce toxin at low temperatures, the potentiation of their toxins by trypsin, and the susceptibility of their spores to heat, as well as their sugar fermentation patterns. Lee and Riemann (10), who studied deoxyribonucleic acid binding homology, have shown that the proteolytic and nonproteolytic strains of *C. botulinum* are genetically heterogeneous. Types C and D form a separate group although they, too, are nonproteolytic. Holdeman and Brooks (Proc. 1st U.S.-Japan Conf. Toxic Microorganisms, in press), on the basis of reactions on 58 substrates, acid and alcohol products, and limited studies of nitrogen metabolism, separated *C. botulinum* into three distinct physiological groups, as follows: Group I, type A and the proteolytic strains of types B and F; Group II, types C and D; Group III, type E and the nonproteolytic strains of B and F.

For complete characterization of the antigenic structures of these organisms, the spore antigens must also be considered. Although the information is limited (15), it appears that the distribution of the spore antigens among the strains differs from that of the somatic antigens. The proteolytic strains of types A and B have different spore antigens, as do the nonproteolytic strains of types E and F.

In the taxonomy of botulinal organisms the antigenic specificity of the common toxin is used to classify together organisms which differ strikingly in their physiology and in their somatic antigens. If it should be confirmed that the spore antigens correspond to the type of toxin produced by these organisms whereas the somatic antigens do not, the decision as to which antigens should be considered for proper classification would be difficult.

There were partial cross-reactions of *C. sporogenes*, *C. tetani*, and *C. histolyticum* with the proteolytic strains of types A, B, and F (Table 5). Such cross-reactions were first reported by Mandia (12). However, by using the fluorescent antibody technique with a variety of strains of *C. sporogenes* some investigators have found such cross-reactions (1, 7); others have not (16). Antisera to the nonproteolytic strains did not cross-react with any of the other *Clostridium* species investigated.

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