DEMONSTRATION OF SHARED ANTIGENS IN THE GENUS *CLOSTRIDIUM* BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY. Antigens prepared from several strains of each of 10 *Clostridium* species were used in an indirect enzyme-linked immunosorbent assay with antisera raised against whole cells of a representative strain from each of the 10 species killed by ultra-violet irradiation. With the exception of *C. cadaveris*, the antisera gave similar results with antigens prepared from all strains of the homologous species. Antigens prepared from 13 other clostridial species were then investigated in an ELISA system with the 10 representative antisera. The results showed many cross-reactions, particularly in the *C. perfringens* group, the *C. difficile/sordelli* group and the *C. botulinum/novyi/sporogenes* group.

INTRODUCTION

Bacteria in the genus *Clostridium* are subdivided into species according to a range of morphological and biochemical criteria that include shape, position of spore, the ability to digest proteins and ferment various sugars and the production of a range of extracellular enzymes and toxins. Several identification schemes are available, e.g. those of Holdeman, Cato and Moore (1977) and Willis (1969), but in practice a number of isolates prove difficult to identify. Many species are closely related, and morphological and biochemical variations occur within strains of the same species and even within a subculture of the same strain. Toxigenic and non-toxigenic variants of the same species occur and some species can be distinguished only by differences in the toxins they produce (Cruickshank *et al.*, 1975).

In human and veterinary medicine, immunological methods have been exploited to detect and identify clostridia. Immunofluorescent antibody (IFA) kits are commercially available for *C. botulinum* types A, C and E, *C. septicum*, *C. chauvoei*, *C. novyi* and *C. tetani* (Wellcome Diagnostics, Dartford, England), for the detection of *C. botulinum* types A, B and F in suspected infant botulism (Hatheway and McCroskey, 1981) and for *C. difficile* in cases of antibiotic associated pseudomembranous colitis (Hubert, Ionesco and Sebald, 1981). Several studies of antigenic relationships in the genus *Clostridium* have been based on agglutination tests (Mandia, 1955) or empirical immunofluorescence reactions (Walker and Batty, 1964; Batty and Walker, 1965; Lynt, Solomon and Kautter, 1971) with a range of antisera raised against heat-killed or

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formalin-killed organisms. More recently, we presented evidence for antigenic relationships between *C. difficile* and *C. bifermentans* and *C. sordelli* (Poxton and Byrne, 1981a) based on surface antigens extracted by EDTA. These were demonstrated by an enzyme-linked immunosorbent assay (ELISA) and crossed immunoelectrophoresis and we were able to provide guidelines for the detection of *C. difficile* by immunotechniques (Poxton and Byrne, 1981b).

The work reported here extends these studies to the majority of medically important species of *Clostridium*.

MATERIALS AND METHODS

Bacterial strains. The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT: *Clostridium bifermentans* NCTC1341; *C. butyricum* NCTC7423; *C. chauvoei* NCTC8070; *C. difficile* NCTC11223; *C. fallax* NCTC8380; *C. histolyticum* NCTC nos. 503, 7123 and 7124; *C. novyi* type A NCTC538, type C NCTC9747 and type D NCTC8350; *C. perfringens* type A NCTC8237, type B NCTC3110, type C NCTC3180 and type E NCTC8084; *C. septicum* NCTC547; *C. sordelli* NCTC8780; *C. sporogenes* NCTC8594; *C. tertium* NCTC541 and *C. tetani* NCTC249. *C. irregularis* NCIB11830 (ATCC25756) was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The strains of *C. botulinum* were from the collection in the Botulinum Laboratory, Unilever Research, Sharnbrook, Bedford: type A, strain nos. 1, 7, 8, 9; type B (proteolytic) strain nos. 25, 26, 27; type B (non-proteolytic) strain nos. 188, 194, 229; type C, strain no. 205; type D, strain no. 154; and type E, strain nos. 34, 118, 126, 250. Other strains included our own laboratory cultures of *C. cadaveris* MPRL34, MPRL506, 50233; *C. irregularis* 4403; *C. nexile* MPRL39; *C. novyi* type A GR2A, GR3A; *C. novyi* type B GR2B; *C. paraputrificum* CP3; *C. perenne* MPRL95; *C. sporogenes* 24, 33; *C. subterminale* MPRL303; and *C. tertium* CT1, CT2. Others were obtained from the following sources: *C. absonum* Ha 9103 and *C. paraperfringens* 2227 from Professor S. Nishida, Kanazawa University, Japan and *C. perfringens* type D, L85 from the late Professor C. L. Oakley, University of Leeds. All strains were fully authenticated by performing the biochemical tests described in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (Holdeman *et al.*, 1977).

Culture of strains and preparation of EDTA extracts. Lyophilised cultures were inoculated into Robertson's Cooked Meat Broth (10 ml) and incubated anaerobically with CO₂ 10% for 16 h at 37°C. These cultures (0.2 ml volumes) were used to inoculate 100-ml volumes of proteose peptone-yeast extract broth (Holbrook, Duerden and Deacon, 1977) which were incubated anaerobically with CO₂ 10% for 16 h at 37°C. Spore formation, as determined by phase contrast microscopy, was negligible under these conditions. Antigens were prepared from the 100-ml cultures by extraction with 10mm EDTA by the method described previously (Poxton and Byrne, 1981a) except that the dialysis step was found to be unnecessary and was omitted.

Preparation of antisera. Antisera were raised in New Zealand White rabbits by intravenous injection of whole, washed, killed bacteria during a 5-week period (Poxton, 1979). The bacteria were killed by ultraviolet irradiation and 10⁹ organisms were used for each injection except for the toxigenic strain of *C. sordelli*, NCTC8780 (10⁶–10⁷ organisms, see Poxton and Byrne, 1981a).

Indirect enzyme-linked immunosorbent assay (ELISA). The EDTA extracts were diluted to contain protein 30 µg/ml in antigen buffer and used to coat wells in flat-bottomed microtitre plates. The ELISA technique was performed as described earlier (Poxton, Brown and Collee, 1982).

Reading and presentation of results. In all titrations, a homologous reaction was included and heterologous reactions were compared directly with this. A reaction was considered significant if the titre was within six doubling dilutions (T/32) of the titre of the reference reaction obtained between a reference antiserum and the homologous antigen preparation. The results are expressed as a fraction of the homologous reference titre. The size of the symbol in the figure is proportional to the degree of the reaction or cross-reaction, on this basis. A reaction at a titre of less than 1/32 of the homologous titre was considered insignificant.

RESULTS

Reference homologous reactions

Initially, EDTA extracts prepared from 10 species of *Clostridium* were used as antigens in an indirect ELISA system with their homologous antisera. During the course of the study, these homologous reactions were included as controls whenever a specific antiserum was used and the range of titres (table) reflects the variation found in repeated titrations.

TABLE
Reactions of EDTA antigens with homologous antisera in an indirect ELISA system

Reference antiserum raised against (species and strain no.)	Range of homologous titres (T)	Number of strains reacting to a titre of							Total number of strains tested
		T	T/2	T/4	T/8	T/16	T/32	<T/32	
<i>C. cadaveris</i> (MPRL34)	12800–25600	1					1	1	3
<i>C. chauvoei</i> (NCTC8070)	12800–25600	1	1						2
<i>C. difficile</i> (NCTC11223)	25600–51200	6	25	3					34
<i>C. histolyticum</i> (NCTC503)	6400–51200	2	1						3
<i>C. novyi</i> , type A (NCTC538)	25600–102400	1				1	1		3
<i>C. perfringens</i> , type A (NCTC8237)	12800–51200	2	4	1					7
<i>C. septicum</i> (NCTC547)	12800–25600	3	2						5
<i>C. sordelli</i> (NCTC8780)	12800–25600	8							8
<i>C. sporogenes</i> (NCTC8594)	6400–25600	1		2					3
<i>C. tertium</i> (NCTC541)	1600–3200	1	2						3

Tests with representative strains

Antigens were prepared from 61 other strains of *Clostridium* belonging to the 10 species listed in the table; they were then used to compare the titres with these antigens and the 10 antisera (now referred to as reference antisera) with those of the homologous reactions. The results indicated that most of the antigens gave titres within one or two doubling dilutions of the homologous reaction (table). Only the antiserum against *C. cadaveris* failed to react significantly with either of the other two strains tested. The reactions of the *C. novyi* type A strains were not as strong as those of the other species but a reaction was considered significant if the titre was within six doubling dilutions (T/32) of that of the homologous reaction (see *Methods*).

Studies of cross-reactions

With the knowledge that nine of the reference antisera reacted strongly with antigens prepared from several strains of the same species, all 10 reference antisera were used to investigate cross-reactions with antigens from at least two strains of 13 other clostridial species, including five different types of *C. perfringens* and *C. botulinum*, and four of *C. novyi*. The results are summarised in the figure. They are expressed as a fraction of the titre obtained with the reference antiserum and the homologous antigen (see *Methods*). Three areas of major cross-reactions were identified: in the *C. perfringens* group, in the *C. difficile/sordelli* group, and in the *C. botulinum/novyi/sporogenes* complex.

FIG.—Cross-reactions within the genus *Clostridium* in an indirect ELISA test with EDTA-extracted antigens and whole cell antisera.

DISCUSSION

The *C. perfringens* group included the first 10 species listed in column 1 of the figure. From previous work, some of the cross-reactions might have been expected, although others were not. All types of *C. perfringens* (A–E) were indistinguishable by

this test and they reacted strongly with *C. chauvoei*, *C. septicum* and *C. tertium* antisera as well as with the *C. perfringens* type A reference antiserum. *C. perfringens*, *C. paraperfringens* and *C. absonum* are related biochemically and antigenic relationships could be expected. *C. chauvoei* and *C. septicum* are also inter-related but it was unexpected to find that these species reacted strongly with the reference antisera against *C. perfringens* and *C. tertium*. The strong reactions of the above species, together with those of *C. fallax* and *C. butyricum* with the first four reference antisera, and to a lesser extent with the *C. cadaveris* and *C. sporogenes* antisera, confirmed the cross-reactions. Antigens prepared from *C. botulinum* type B (non-proteolytic) and type E were also included in this group.

The *C. sordelli/bifermentans* and *C. difficile* group were studied in detail before (Poxton and Byrne, 1981a) but in this extended survey we included the species *C. irregularis*. We have been able to obtain only two strains of *C. irregularis*, one isolated in our own laboratory, strain 4403, and one from the NCIB (strain no. 11830, which is the same as ATCC25756). Both strains were indistinguishable from *C. difficile*, *C. sordelli* and *C. bifermentans* in our ELISA system and shared the same major carbohydrate antigens as these strains (I. R. Poxton, unpublished results). The antigens from these four species did not cross-react with any other antisera, nor did the *C. sordelli* or *C. difficile* antisera cross-react with any other antigen.

It is well established that organisms in the *C. botulinum/novyi/sporogenes* complex possess some biochemical and cultural similarities (Smith, 1977). *C. botulinum* type A and type B (proteolytic) are biochemically and culturally indistinguishable from *C. sporogenes*; only the toxins separate them. This relationship was confirmed by the ELISA technique which showed the presence of shared antigens. Three of four type-A strains and all of three proteolytic type-B strains reacted with the *C. sporogenes* reference antiserum. Similarly, *C. botulinum* types C and D are indistinguishable from *C. novyi* type A (except by toxin), and this was borne out in this study. The unexpected result in this group was the demonstration of cross-reactions between *C. botulinum* type B (non-proteolytic) and type E antigens with the first four reference antisera. Taken in isolation this result may be questioned; however, non-proteolytic type B and type E *C. botulinum* are distinguished only by toxin production (Smith, 1977) and all of three strains of non-proteolytic type B and all of four strains of *C. botulinum* type E gave identical results with the four reference antisera, indicating that the result was consistent.

The antisera prepared from *C. sporogenes* and *C. cadaveris* antigens had remarkably similar patterns of specificity. The *C. cadaveris* antiserum, however, may be atypical as antigens prepared from the other two strains did not react significantly with it.

This study has demonstrated some unexpected shared antigens in the genus *Clostridium* but has confirmed others. The EDTA preparation used as antigen for the *C. difficile/sordelli* group contained proteins and carbohydrate extracted from the cell surface (Poxton and Byrne, 1981a; Poxton and Cartmill, 1982); the previously unrecognised cross-reactions demonstrated in this study were presumably due to the presence of heat- and formaldehyde-labile antigens not detected in earlier studies. It is difficult to compare our results with those obtained in previous studies which have been used to separate clostridia by immunofluorescent antibody techniques, for such tests to be satisfactory they must be specific, and cross-reacting antibodies were removed by

absorption. The assay system we have used is relatively non-selective and highly sensitive. We suggest that the results presented here may provide useful guidelines when designing immunological methods for the detection of clostridia and may also help to define taxonomic relationships in the genus. A detailed investigation at the molecular level of some of the more important areas of cross reactions is currently being made.

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