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Antigenicity of Converting Phages Obtained from *Clostridium botulinum* Types C and D

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Phage conversion of toxigenicity in *Clostridium botulinum* types C and D was accomplished by using nontoxigenic strains and phages purified from plaques. Although the morphology of the converting phages seemed to be the same, they were divided into three groups on the basis of their conversion spectrum. The first group consists of phages obtained from toxigenic strains C-Stockholm and C-468. The second group consists of phages from strains D-1873 and C-203. The third group consists of phages from strains D-South African and D-4947. These converting phages were also classified into the same three groups by a neutralization test with specific antiphage sera. Cross-neutralization, however, was observed between phages belonging to group 1 and group 2, by both the neutralization test of converting ability and by a plaque experiment in which the surviving rates of phages were calculated after treatment with each antiphage serum. The antigenic differences among these converting phages should probably comprise one of the reasons for the existence of the specific infection spectrum in *C. botulinum* types C and D.

We reported that some nontoxigenic strains of *C. botulinum* types C and D were converted to a toxigenic state when they were mixed with the filtrates of induced lysates of toxigenic strains. A complete phage was demonstrated in each filtrate (5), and it was strongly suggested that this phenomenon was phage conversion (6-8). Eklund et al. succeeded in making plaques on a solid medium and verified and extended the above findings (1-3).

Recently, we also obtained phages purified from plaques by following the procedure reported by Eklund et al., and a conversion experiment was carried out with these purified phages. Some nontoxigenic strains were converted to a toxigenic state by the specific bacteriophages, whereas the other strains were not infected by these phages. This report deals with the antigenicity of converting phages in order to clarify the basis for the specific infection spectrum shown by these phages.

MATERIALS AND METHODS

Strains and media. Toxigenic strains D-South African (D-SA Tox) and D-4947 were kindly provided by D. A. Boroff, Einstein Medical Center, Philadelphia. The nontoxigenic strain D-South African (D-SA NT) was isolated spontaneously from the D-SA Tox. The other strains employed were reported in a previous article (8). These strains were stored in cooked meat medium and transferred into a medium containing 1% lactalbumin, 2% yeast extract, 0.5% glucose, and 0.15% cysteine hydrochloride, pH 7.2 (LYG), when required.

Plaque formation. Many phages were induced from the toxigenic strains by mitomycin C treatment (1 μg/ml) as reported previously (5). The induced lysates were filtered through a membrane filter (pore size, 450 nm). Plaque formation was carried out by the procedure reported by Eklund et al. (1-3), except that brain heart infusion agar (1.5%) was used as the basal medium in all cases and that LYG soft agar (0.8%) was used as an overlay in D-SA NT instead of Trypticase-peptone-glucose-yeast extract medium. With the other indicator strains, soft agar prepared from Segners fortified egg meat medium was used as the overlay. A portion of each phage preparation, 0.1 ml, and 0.5 ml of each young indicator strain culture were inoculated into 3 ml of overlay soft-agar medium. At the same time, human blood, 0.2 ml, NaCl at a final concentration of 0.2%, and catalase (Sigma, 3,600 U/ml), 500 μg/ml, were added to these overlay media as reported by Eklund et al. Immediately after inoculation, the preparations were plated and incubated at 37 C for 18 h anaerobically. The phages obtained by five successive single-plaque isolations are shown in Table 1. These purified phages were passaged several times in suitable host strains to increase the phage titer and then used in the following experiments.

Antiphage sera. Anti-c-468, anti-d-1873, and anti-d-sA phage sera were prepared by the following methods. Each 2 liters of passaged phage was clarified by centrifugation (8,000 rpm, 30 min) and sterilized by filtration. These filtrates were concentrated

TABLE 1. *Phages, indicator strains, and overlay media*

Phage	Source	Indicator strain	Overlay medium
	Toxigenic	Nontoxigenic	
c-st	C-Stockholm	C-AO2	SFEM[a]
c-468	C-468	C-AO2	SFEM
d-1873	D-1873	D-151	SFEM
c-203	C-203	D-151	SFEM
d-sA	D-South african (D-SA Tox)	D-SA NT	LYG[b]
d-4947	D-4947	D-SA NT	LYG[b]

[a] SFEM, Segners fortified egg meat medium; human blood added.
[b] NaCl and catalase added.

by polyethylene glycol from 2 liters to 90 ml at 4 C and then dialyzed against 0.01 M potassium phosphate-buffered saline (pH 7.2) at 4 C overnight. The concentrated phages thus obtained were partially purified by low-speed centrifugation (10,000 rpm, 30 min) and ultracentrifugation (60,000 × *g*, 180 min), alternately performed three times, to minimize the toxin associated with phage preparations. Finally, each 2.5 ml of phage was resuspended in 0.01 M phosphate-buffered saline. These phages were mixed with an equal volume of Freund incomplete adjuvant and injected into two rabbits subcutaneously. The second injection of each phage preparation, prepared by the same procedure, was carried out on day 21 after the first injection. Using the same time interval, each rabbit was injected by the intraveneous route with 5 ml of phage preparation obtained by ultracentrifugation (70,000 × *g*, 180 min) from 500 ml of passaged phages. Before this injection, the rabbits had received 10 U of rabbit antitoxin serum to protect the animals against toxin in the inoculum (4). The rabbits were bled on day 3 after the last injection. The antisera obtained were inactivated at 56 C for 30 min and then absorbed by the respective indicator cell suspensions. These sera neutralized the homologous phages, which had a converting titer of 10^{-6} at a dilution of 1:64 or 1:32 when the conversion test was carried out, after serial-twofold-diluted serum was mixed and incubated with an equal volume of phage at 37 C for 2 h.

Conversion and neutralization tests. The conversion experiment was carried out by mixing 0.5 ml of passaged phage filtrate and an equal volume of young, nontoxigenic strains in 2 ml of LYG medium. After incubation at 37 C for 4 h, 0.2 ml of each culture was transferred into 10 ml of cooked meat medium and incubated at 37 C for 3 days. The supernatants of culture fluids were diluted 10 times with gelatin buffer (pH 6.0) and tested for the toxicity by injecting 0.5 ml into mice intraperitoneally.

Neutralization testing of phages was done by the following method. A portion, 0.1 ml, of phage diluted serially in 10-fold dilution with LYG medium was mixed with 0.9 ml of 10-times-diluted antiphage serum. Control phage was mixed with 10-times-diluted normal rabbit serum instead of antiserum. After incubation at 37 C for 2 h, 0.5 ml was pipetted from this mixture and used for the conversion test. The surviving rate was obtained by mixing 0.1 ml of phage and 0.9 ml of antiphage serum, both diluted 1:10, at 37 C. Each 0.1 ml was pipetted from the mixture at intervals of 5, 10, 20, and 30 min and plated with the suitable host strain. The number of plaques demonstrated was counted and the surviving rate was calculated.

RESULTS

Morphology of phages and plaques. From the induced lysates of toxigenic strains, a group of phages was obtained. The morphology and the size of these phages seemed to be the same as those reported previously (5).

Differences in plaque morphology of phages c-468, c-st, c-203, and d-1873 were slight. Plaque size was about 1 to 3 mm in diameter, and clear plaques were always observed (Fig. 1). The colony-centered plaque reported by Eklund et al. was not obtained in our experiment. These phage preparations that were passaged through indicator strains for several times contained about 10^8 plaque-forming units (PFU)/ml. However, turbid plaques, 0.5 to 3 mm in diameter, were demonstrated by phage d-sA. This indicates a possible reason why lysis of strain D-SA NT by phage d-sA was not as obvious as it was with the other combinations of indicator strains and phages. Thus, the number of plaques obtained by d-sA was about 10^6/ml, and the converting titer was also about 10 times lower than with the other phages (see Table 4). In the case of the d-4947 phage, we have not yet succeeded in obtaining lytic phage. Only a few turbid plaques were observed when the filtrate of the mitomycin C-induced lysate of the strain D-4947 culture was plated with the indicator strain D-SA NT. The morphology of this phage seemed to be the same as the d-sA phage.

Conversion spectrum. Various nontoxigenic strains were mixed with phages purified from plaques, and the conversion test was carried out. As shown in Table 2, some of the nontoxigenic strains were converted to a toxigenic state by specific bacteriophages. The converting phages were divided into three groups on the basis of their infection spectrum, which was exactly the same as that reported with crude phage preparations (10). As expected, the cells isolated from plaques had been converted to a toxigenic state.

Antigenicity of converting phages. A portion of phages, 0.1 ml, and 0.9 ml of antiphage sera, both diluted 10 times, were mixed and incubated at 37 C for 2 h, and then the conversion test was carried out. From the results shown in Table 3, the converting phages were

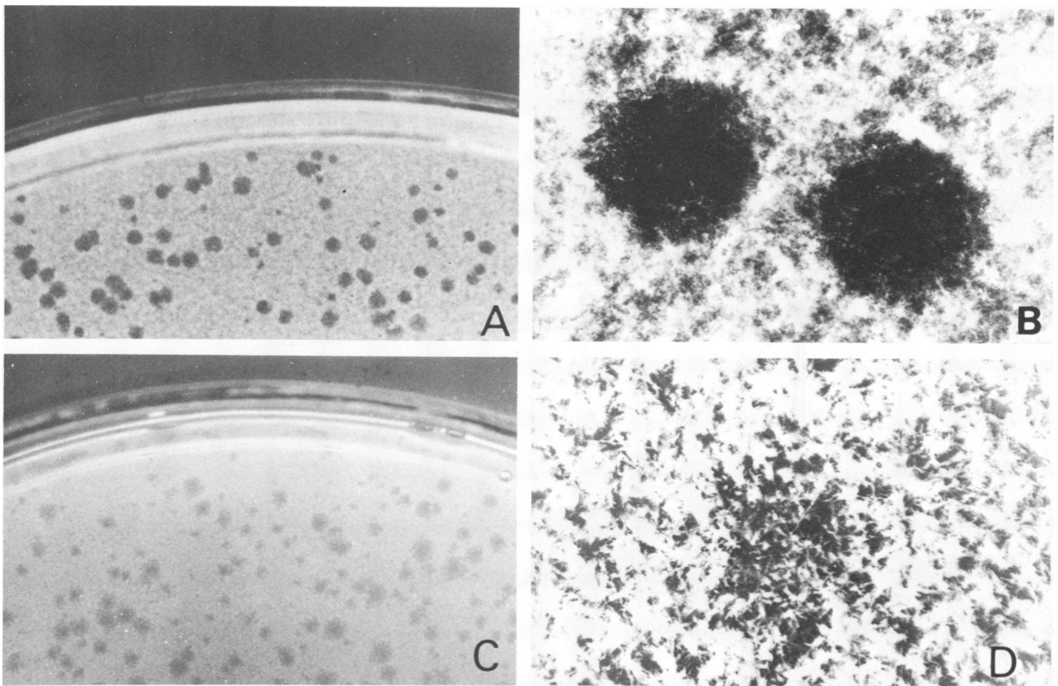

FIG. 1. *Plaques of type C and D phages. (A, B) plaques formed by c-st phage on indicator strain C-A02; (C, D) plaques formed by d-sA phage on indicator strain D-SA NT; (B, D) plaques under the microscope (ca. ×10).*

TABLE 2. *Conversion of toxigenicity with various nontoxigenic strains and phages*

Nontoxigenic strain	Phage					
	c-468	c-st	c-203	d-1873	d-sA	d-4947
C-AO2	C	C				
C-468-U31	C	C				
C-203-U28						
C-203-MC1						
C-203-AO1						
C-203						
C-6813						
C-N71						
D-134			C	D		
D-151			C	D		
D-139	C	C				
D-SA					D	D

[a] The type of toxin was determined by neutralization testing by mixing 10 minimal lethal doses of each toxic culture and antitoxic serum of types C and D, distributed by the Center for Disease Control, Atlanta, Ga.

also classified into three groups on the basis of their antigenicity. To confirm the existence of cross-neutralization between these phages, the neutralization test was carried out with 10-times-diluted antisera and phages diluted in serial 10-fold dilutions. The results were summarized in Table 4. The converting titers of phages belonging to group 1 and group 2 were not decreased by anti-d-sA phage serum. Also, the converting ability of phage d-sA was not neutralized by anti-c-468 and anti-d-1873 phage sera. Cross-neutralization, however, was shown between phages belonging to group 1 and group 2. Furthermore, surviving rates of these phages were calculated. As shown in Fig.

TABLE 3. *Neutralization test of phages by antiphage sera*[a]

Phage	Group	Antiphage serum		
		c-468	d-1873	d-sA
c-468	1	s[b]	d	d
c-st		s	d	d
d-1873	2	d	s	d
c-203		d	s	d
d-sA	3	d	d	s
d-4947		d	d	s

[a] Phages and antisera, both diluted 1:10, were mixed and incubated at 37 C for 2 h, and then the conversion test was done.

[b] s, Survived; d, died.

2 and 3, the phages c-468 and c-st were neutralized by anti-c-468 phage serum to the same extent. Also, phages d-1873 and c-203 exhibited the same survival rate with anti-d-1873 phage serum. Cross-neutralization, however, was observed between these two groups of phages. The number of plaques formed by these four phages was not decreased by anti-d-sA phage serum. It was also observed that the phage d-sA (6 × 10^5 PFU/ml) was neutralized completely by anti-d-sA phage serum after incubation at 37 C for as little as 10 min. On the other hand, the same phage, 4 × 10^5 PFU/ml and 3 × 10^5 PFU/ml, was demonstrated after treatment at 37 C for 30 min with anti-c-468 and anti-d-1873 phage sera, respectively.

DISCUSSION

The antigenicity of converting phages purified from plaques was investigated. Although

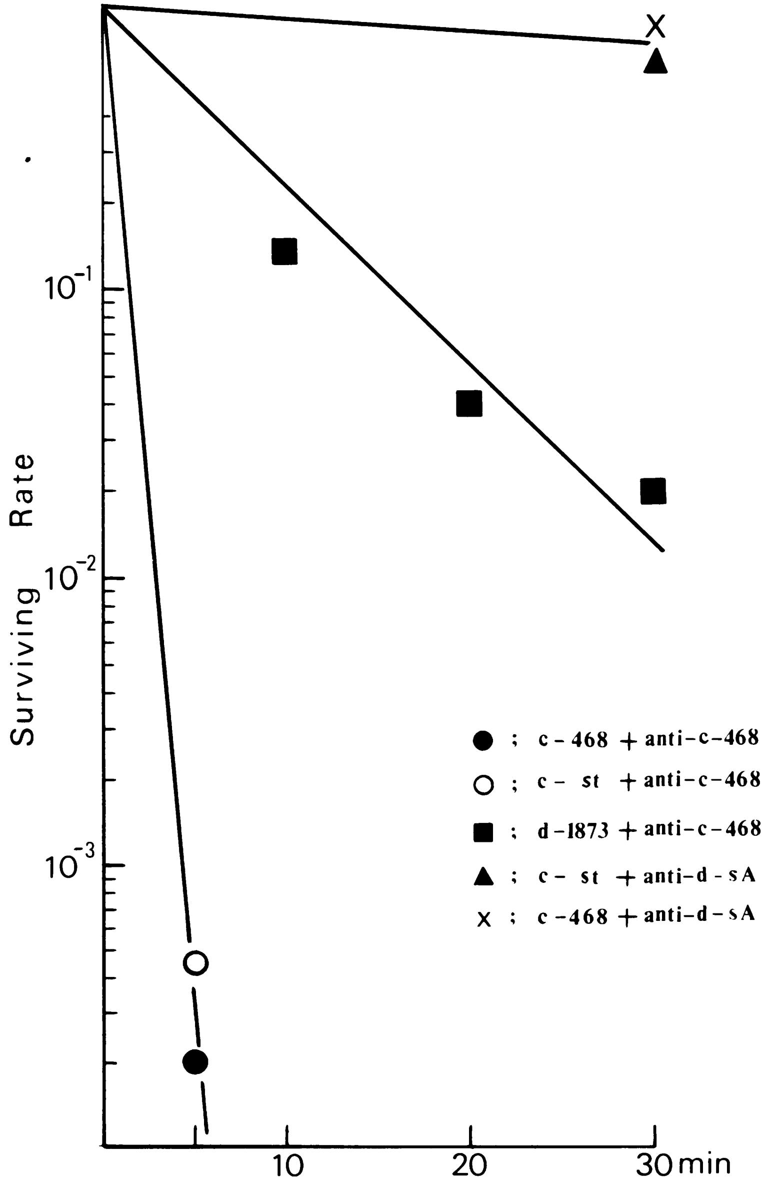

FIG. 2. *Neutralization curves with anti-c-468 and anti-d-sA phage sera. Phages and antisera, both diluted 1:10, were mixed and incubated at 37 C. Surviving rate was calculated at 5, 10, 20, and 30 min.*

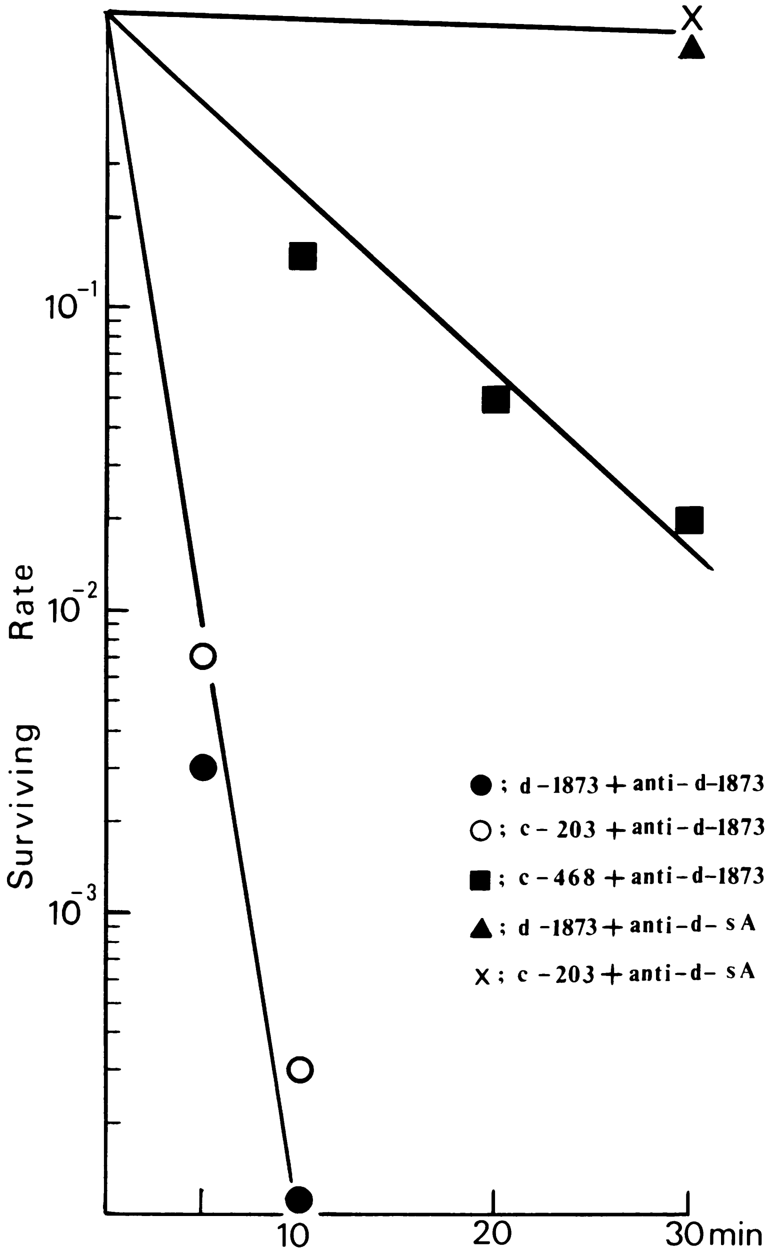

FIG. 3. *Neutralization curves with anti-d-1873 and anti-d-sA phage sera.*

the morphology of converting phages seemed to be the same, they were divided into three groups on the basis of their conversion spectrum. From the results of the neutralization test, it was clear that the antigenicity of phages belonging to the same group was identical, though cross-neutralization was observed between phages belonging to group 1 and group 2. When the indicator strains, C-A02 and D-151, were infected by phages d-1873 and c-468, respectively, no plaques appeared. Therefore, the explanation behind the fact that only the specific phages were able to infect and convert the specific nontoxigenic strains is most likely the antigenic differences among converting phages.

The converting phages d-1873 and c-203 were demonstrated to have the same antigenicity and infection spectrum, though the type of

TABLE 4. *Further neutralization testing of phages by antiphage sera*[a]

Phage	Converting titer			
	Control	Treated with antiphage serum		
		c-468	d-1873	d-sA
c-468	10^{-7}	s[b]	10^{-5}	10^{-7}
c-st	10^{-7}	s	10^{-5}	10^{-7}
d-1873	10^{-7}	10^{-5}	s	10^{-7}
d-sA	10^{-6}	10^{-6}	10^{-6}	s

[a] Each phage diluted in serial 10-fold dilution was treated with 10-times-diluted antisera or normal rabbit serum. After incubation at 37 C for 2 h, the conversion test was carried out, and the converting titer of each phage was obtained.

[b] s, Survived (neutralized completely).

toxin determined by these two phages was different. This strongly suggests that these two phages are the same except for the gene related to the toxin production.

The nontoxigenic strain D-139, isolated from the toxigenic strain D-1873 by the treatment with acridine orange, was converted to produce type C toxin by phages c-468 and c-st. The nontoxigenic strains D-134 and D-151 isolated from D-1873 by the same procedure were converted to produce type D toxin by phage d-1873 and to produce type C toxin by phage c-203. From these results, we presume that there may be two specific receptors in strain D-1873, one for phages c-468 and c-st, and the other for phages d-1873 and c-203. Therefore, the noninfectivity observed in some combinations of phages and host organisms seems to be due to the absence of the specific receptor(s) in the organism. On the other hand, we have reported that nonconverting phages that showed the same antigenicity as that of parent converting phages were obtained (7a, 9). The indicator strains infected by these nonconverting phages acquired immunity to the infection by the parent phages. This result suggests an alternative explanation for the fact discussed above: some nontoxigenic strains might have been lysogenized by a phage that has a defect in the gene controlling toxin production. The studies necessary for clarification of these points are being carried out at the present time.

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LITERATURE CITED

1. **Eklund, M. W., F. T. Poysky, S. M. Reed, and C. A. Smith.** 1971. Bacteriophages and toxigenicity of *clostridium botulinum* type C. Science 172:480-482.
2. **Eklund, M. W., F. T. Poysky, and S. M. Reed.** 1972. Bacteriophage and the toxigenicity of *Clostridium botulinum* type D. Nature (London) New Biol. 235:16-17.
3. **Eklund, M. W., and F. T. Poysky.** 1974. Interconversion of types C and D strains of *Clostridium botulinum* by specific bacteriophages. Appl. Microbiol. 27:251-258.
4. **Groman, N. B., and M. Eaton.** 1955. Genetic factors in *Corynebacterium diphtheriae* conversion. J. Bacteriol. 70:637-640.
5. **Inoue, K., and H. Iida.** 1968. Bacteriophages of *Clostridium botulinum*. J. Virol. 2:537-540.
6. **Inoue, K., and H. Iida.** 1970. Conversion of toxigenicity in *Clostridium botulinum* type C. Jpn. J. Microbiol. 14:87-89.
7. **Inoue, K., and H. Iida.** 1971. Phage-conversion of toxigenicity in *Clostridium botulinum* types C and D. Jpn. J. Med. Sci. Biol. 24:53-56.

7a. **Oguma, K.** 1976. The stability of toxigenicity in Clostridium botulinum types C and D. J. Gen. Microbiol. 92:67-75.

8. **Oguma, K., H. Iida, and K. Inoue.** 1973. Bacteriophage and toxigenicity in *Clostridium botulinum:* an additional evidence for phage conversion. Jpn. J. Microbiol. 17:425-426.
9. **Oguma, K., H. Iida, and K. Inoue.** 1975. Observations on nonconverting phage, c-n71, obtained from a nontoxigenic strain of *Clostridium botulinum* type C. Jpn. J. Microbiol. 19:167-172.
10. **Oguma, K., H. Iida, and K. Inoue.** 1975. Bacteriophages and toxigenicity in *Clostridium botulinum* types C and D. Jpn. J. Med. Sci. Biol. 28:63-66.