MITOMYCIN-INDUCED LYSIS OF CLOSTRIDIUM SORDELLII

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PLATE VIII

We recently found that broth cultures of Clostridium sordellii lysed readily when mitomycin C was added during the logarithmic phase of growth; a lytic agent active on the cells of C. sordellii was released. In view of the significant role of lysogeny on toxigenicity of strains of C. botulinum (Inoue and Iida, 1970; Eklund, Poysky and Reed, 1972), we attempted to establish a correlation between toxigenicity and susceptibility to mitomycin C, which in turn is closely related to lysogeny, in studies with a number of toxigenic and non-toxigenic strains of C. sordellii. This species was chosen for the study because a number of non-toxigenic strains of C. sordellii have been described (Brooks and Epps, 1958; Novotoný, 1969), although identification of non-toxigenic strains belonging to a pathogenic species of Clostridium is extremely difficult.

MATERIALS AND METHODS

Strains. Toxigenic strains of C. sordellii include nos. 1734, 4707, 4708, 3703, 1623, 1733, SJ4 and 82-P. Non-toxigenic strains include nos. 1340, 1619, 1620, 6800, 6929, 1621, 4709, 7222R and 1734HT. All of the above-mentioned strains except nos. 82-P, SJ4, 7222R and 1734HT were received from Mrs Irene Batty, Wellcome Research Laboratories, England, in 1961. Other non-toxigenic strains, nos. IV-1, IV-3, IV-7, IV-11, IV-12, IV-13, IV-14, IV-22, IV-25 and IV-40 were received from Dr E. A. Lozano, Montana State University, Montana, USA, in 1970. C. sordellii strain 82-P is originally the same strain as C. sordellii 82 from which SJ2, SJ3 and SJ4 were obtained by single-cell isolation by Dr K. I. Johnston, Leeds University, England and Dr C. T. Huang, Hong Kong University, Hong Kong (Huang, Tamai and Nishida, 1965). The strain 82 stocked in our laboratory is non-toxigenic and urease-negative at present. The strain 82-P was provided to our laboratory, by Dr A. R. Prévot, Pasteur Institute, Paris. Strain 7222R was brought by Dr C. T. Huang. This strain, although still urease-positive, has lost its toxigenicity at present. Strain 7222HT is a non-toxigenic urease-negative substrain recovered after repeated heating of the culture of the parent strain 7222R (Huang, Tamai and Nishida, 1965).

C. perfringens strain 006-1 and C. sporogenes strain no. 1 were used as reference strains.

Medium. PYF medium consisted of Proteose Peptone no. 2 (Difco) 2.0% (w/v); yeast extract (Daigo, Osaka, Japan) 0.5%; fructose 1.0%; sodium chloride 0.5%; and sodium thioglycollate 0.1% (pH 7.2). This medium, unless otherwise stated, was used throughout the present investigation.

Susceptibility to mitomycin C. A 0.2-ml volume of a bacterial culture grown in PYF medium for 15 hours was transferred into 10 ml of PYF medium in a tube (2×15 cm) and was incubated at 37°C until the optical density at 560 nm (OD560) of the bacterial growth

reached 0-1 to 0-15. Then mitomycin C (MC; Sankyo Co., Tokyo, Japan) was added to a final concentration of 0-5 or 1-0 \( \mu \)g per ml of culture. The effect of MC was recorded as positive when bacterial lysis sufficient to reduce the OD\(_{560}\) reading by 0-1 or more occurred in the culture within 3 hours after the addition of the drug. Optical density was measured in a Shimazu Bosch-Lomb Spectronic 20 spectrophotometer (Shimazu Co., Kyoto, Japan). Results were confirmed by repeating each experiment at least once. Other drugs used were chloramphenicol (Sankyo Co., Tokyo, Japan), tetracycline (Japan Lederle, Tokyo, Japan), cycloserine (Meijiseika, Tokyo, Japan) and penicillin (Takeda, Osaka, Japan). 4-Nitroquinoline 1-oxide (4NQO) was kindly provided by Dr M. Inuzuka, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan.

Preparation of lysates. For preparation of MC-induced lysate (MC lysate), MC was added to the culture as detailed above. An extract was prepared by sonic disintegration of cells grown in PYF medium and collected during the logarithmic phase; the cells were treated in a Tominaga sonicator (Tominaga, Tokyo, Japan) at 20 Kc for 10 min. Autolysate was prepared from 48-hour cultures grown in PYF medium in which Polypeptone (Daigo, Osaka, Japan) was used in place of Proteose Peptone no. 2 because autolysis readily occurred in the presence of Polypeptone. These lysates and the sonic extracts were centrifuged at 3000\( \times \)g for 10 min. to remove cellular debris and their supernates were used as cell-free lysates and cell-free extracts respectively. The cell-free MC lysate was fractionated into supernatant fraction (phage-free lysate) and pellet by centrifugation at 100,000\( \times \)g for 90 min. in a Hitachi 65P Automatic Preparative Ultracentrifuge (Hitachi, Tokyo, Japan). The pellet was suspended in PYF medium and the suspension could be stored for at least 2 weeks without loss of the lytic activity.

Preparation of indicator cells for lytic agent. For preparation of freeze-thawed cells (FT cells), a 10-ml volume of a 15-hour culture in PYF medium at 37°C was harvested by centrifugation, resuspended in 1 ml of 0-05M-tris HCl buffer (pH 7-4) and was immediately frozen at -20°C. Vegetative cells were prepared from PYF cultures in the logarithmic phase at 37°C. The culture was rapidly cooled in ice, harvested by centrifugation, resuspended in 0-05M-tris-HCl buffer (pH 7-4) and was immediately tested. Heated cells were prepared by heating the above-mentioned suspension at 60°C for 10 min.

Detection of lytic agent. FT cells with or without lysate were incubated at 37°C. Decrease of the OD\(_{560}\) reading was estimated every 5 min. and the result was read after incubation for 30 min. Tests for presence of lytic agent were recorded as positive when the difference in OD between FT cells with and without lysate was more than 0-2.

Electron microscopy. The above-mentioned pellet fraction obtained by ultracentrifugation at 100,000\( \times \)g for 90 min. was suspended in neutral 0-1M ammonium acetate solution. Specimen grids for electron microscopy were prepared with the pellet suspension and drained on filter paper. The sample was stained with a 2% solution of phosphotungstic acid (pH 6-0-7-0), and was dried on filter paper. An electron microscope type-7 (Japan Electron Optics Laboratory Co., Tokyo, Japan) with a direct magnification of 20,000 was used.

RESULTS

Mitomycin-induced lysis

A preliminary study disclosed that \textit{C. sordellii} strains could be lysed by irradiation with ultraviolet light (UV) or by treatment with MC. This finding prompted us to test our \textit{C. sordellii} strains for lysogeny. In view of the experimental difficulties inherent in attempts to expose anaerobes to UV without exposure to air, we employed MC in the present test for lysogeny. Broth cultures of 24 out of 28 \textit{C. sordellii} strains, including toxigenic and non-toxigenic strains, lysed when MC was added at a final concentration of 1-0 \( \mu \)g per ml during their early stage of logarithmic growth (fig. 1). Cell lysis began within 30 to 60 min. of growth after the addition of MC and was completed.
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within the ensuing 3 hours. A certain difference could be found between toxigenic and non-toxigenic strains when MC was used at a concentration of

![Graph](image)

**Fig. 1.**—Lysis of *Clostridium sordellii* by addition of mitomycin C (MC); ○—○ MC content 1·0 μg per ml; △—△ MC content 0·5 μg per ml; ⧫—○ control (no addition of MC). Strains used in fig. 1 are *C. sordellii* nos. 1734, 1340 and 6929 respectively from left to right.

0·5 μg per ml in place of 1·0 μg per ml. Representative results given in the table suggest that toxigenic strains were more susceptible than the non-toxigenic strains to the drug.

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<th>Susceptibility to mitomycin C (MC) of toxigenic and non-toxigenic strains of Clostridium sordellii</th>
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Single-cell isolates, strains SJ2, SJ3 and SJ4 obtained from a parent *C. sordellii* strain 82, exhibited different reactions when exposed to MC; the non-toxigenic and urease-negative strain SJ3 was not susceptible to MC at a concentration of 0·5 μg or 1·0 μg per ml, whilst strain SJ2, also non-toxigenic and urease-negative, was not susceptible to MC at 0·5 μg per ml but was susceptible to 1·0 μg per ml. Another strain—SJ4—which is toxigenic and urease-positive, was susceptible to MC at both concentrations.
Phage-like particles in lysates

To detect phage or phage-like particles in MC lysates of toxigenic and non-toxigenic strains of *C. sordellii*, the lysates were centrifuged at 3000g for 15 min. and the resulting supernatates were then centrifuged at 100,000g for 1.5 hours to produce pellets for this study. Phage-like particles with different morphologies were demonstrated by electronmicroscopic examination of the pellets prepared from all strains examined (fig. 2). Plaque formation, however, could not be demonstrated when suspensions of pellets obtained from the lysate of strain 1734 were spotted on lawns of 11 strains of *C. sordellii*.

Phage-like particles could be demonstrated also in a 48-hour culture autolysate of strain 82-P, although the number of phage-like particles was much fewer in the autolysate than in MC lysate (fig. 3). Phage-like particles were not observed in autolysates of another toxigenic strain and two non-toxigenic strains tested.

The lytic agent

When MC lysate of *C. sordellii* strain 1734 was separately added to vegetative, heated and FT-cell suspensions of *C. sordellii*, lysis was most marked with the FT cells, less marked with the heated cells and not demonstrable with the vegetative cells (fig. 4). A cell suspension of *C. sordellii* strain 6929 was used as the indicator strain in this study because the strain exhibited the least autolytic character among the strains that we examined. The activity
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**Fig. 2.**—Phage-like particles in MC lysate; (a) *Clostridium sordellii* no. 1734, (b) *C. sordellii* no. 7222R, (c) *C. sordellii* no. 4707, (d) *C. sordellii* no. 82-P and (e) *C. sordellii* no. 1620. × 100,000.

**Fig. 3.**—Phage-like particles in autolysate of *Clostridium sordellii* no. 82-P. × 100,000.
of the MC lysate of strain 1734 against strain 6929 was not reduced by centrifugation at 100,000g for 90 min. and the resultant pellet had only slight lytic activity. Autolysate and sonic extract of strain 1734 also had lytic activity against strain 6929 (fig. 5). MC lysates of two other C. sordellii strains 7222\R and SJ4 were examined for lytic activity against strain 6929. Findings similar to those noted above were obtained with the MC lysates of strain 7222\R, but the MC lysate of SJ4 was distinctly weak in its lytic activity.

![Graph showing lysis of FT cells of Clostridium sordellii no. 6929 by MC-lysate fractions, cultural autolysate and sonic extract of C. sordellii no. 1734; MC lysate ○○; supernatant △△ and pellet ×× fractions of MC lysate; cultural autolysate △△; sonic extract ○○ and control ●●.](image)

**Effects of other antibiotics and mutagenic agents**

Cycloserine, chloramphenicol, tetracycline, or 4NQO at graded concentrations of 1, 10, 50 or 100 μg per ml respectively or penicillin at 1, 10, 50 or 100 units per ml respectively were separately added to cultures of strain 1734, as described in the studies with MC. Cycloserine, chloramphenicol and 4NQO did not give rise to cell lysis, whereas tetracycline caused gradual lysis. Penicillin at a concentration of 1 unit per ml caused rapid lysis. The lytic agent encountered in MC lysate, however, could not be detected in any of the penicillin or tetracycline-induced lysates.

**DISCUSSION**

Broth cultures of 24 of our 28 urease-positive strains of C. sordellii lysed readily when they were treated with MC. Brooks and Epps (1958) reported that there were several known non-pathogenic substrains derived from C. sordellii strain 82. We used the non-toxigenic and urease-negative substrains, SJ2 and
SJ3, but there may be a taxonomic argument against regarding the strains SJ2 and SJ3 as true derivatives of _C. sordellii_. We stock another non-toxigenic and urease-negative strain, 7222HT, which was obtained from cultures of the toxigenic and urease-positive strain 7222R after heating. Strain 7222HT was not susceptible to either of the MC concentrations used, whilst strain 7222R, although it has lost its toxigenicity and was not susceptible to MC at a concentration of 0.5 μg per ml in the present investigation, was susceptible to MC at 1.0 μg per ml.

A mutagenic agent, 4NQO, which was demonstrated to be a phage-inducer by Yamamoto, Fukuda and Takebe (1970), did not seem to be as effective as MC and UV for the phage induction of _C. sordellii_.

Kiritani _et al._ (1973) also stated that a non-toxigenic substrain, NIH–5, obtained from _C. botulinum_ type B, strain NIH, was not susceptible to MC, whereas the toxigenic substrain NIH–19 obtained from the same parent strain was susceptible to the drug. These findings imply that lysogeny is closely related with toxigenicity of clostridia. However, the decisive identification of non-toxigenic variants of toxigenic species of clostridia presents problems.

The lytic agent released into culture medium was different from phage in several aspects; the agent could not be deposited by ultracentrifugation at 100,000g for 1½ hours, could not produce plaques against indicator strains, although it readily lysed the lawns of indicator strains spotted by the agent, and was more active on FT cells than on vegetative cells of _C. sordellii_. Mitsui, Kiritani and Nishida (1973) demonstrated the presence of a lytic agent as well as of phage-like particles in MC lysate of a _C. botulinum_ strain, and Kiritani _et al._ (1973) used the _C. botulinum-C. sporogenes_ group-specific lysin for taxonomic purposes. The lytic agent of _C. sordellii_ described in the present paper also seems to have taxonomic usefulness. Because the lytic agent that we described was definitely less active on heated cells than on FT cells, and because most of the _C. sordellii_ strains were readily subject to autolysis, the rapid lysis of FT cells of _C. sordellii_ by MC lysate seems to be the result of co-ordination of the lytic agent with autolytic enzymes inside the cells.

**Summary**

Broth cultures of 24 out of 28 urease-positive strains of _Clostridium sordellii_ tested lysed readily when mitomycin C (MC) was added early in the logarithmic phase of growth. Toxigenic strains of the species were more susceptible than the non-toxigenic strains to the effect of the drug. Phage-like particles with different morphologies could be obtained from cultures of all of five _C. sordellii_ strains studied. The lytic agent or agents active on freeze-thawed cells of _C. sordellii_ strains were also found in the MC lysates.

We wish to thank Dr E. A. Lozano for a supply of strains of _C. sordellii_, Mr M. Ito for help with the electronmicroscopy and Dr C. T. Huang for helpful comments.

**REFERENCES**

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