Avirulent Clostridium perfringens Strains Obtained by Euflavine Treatment

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Clostridium perfringens was incubated in the presence of euflavine (EU); the resistant mutants which were thus isolated had highly reduced capacity to release α-toxin. This fact was confirmed by lecithinase determinations and by immunoelectrophoresis. Injected into guinea pigs and into 6- to 7-day-old chicks, these mutants were completely avirulent. A study of their properties indicated biochemical differences between wild types and mutants.

Up to this time, very few attempts have been made to study the genetics of anaerobic bacteria. Our work deals with mechanisms of the hereditary transmission of virulence in Clostridium perfringens, the main agent of gas gangrene. Most authors believe that the most important factor for the pathogenicity of the gas bacillus lies with α-toxin. It is now well established that α-toxin (lecithinase C) hydrolyzes various phospholipids of the cell membrane, thereby causing destruction of the cell (6). Although its mechanism of action is chemically known (5), the intimate mechanism of synthesis of this toxin still remains a complete mystery.

During the past few years, several workers have reported that acridine derivatives may eliminate or “wash” the epimones contained in the bacterial cell (4). In bacteria, the properties that are most likely to be carried by an epimone are usually not strictly essential to the life of the cell but are auxiliary to the normal bacterial metabolism.

The purpose of this study was to determine whether the capacity of C. perfringens type A to synthesize α-toxin may be altered by euflavine (EU) and to determine what effects may be expected when the EU-treated cultures are injected into animals.

Materials and Methods

Organisms. C. perfringens type A, strains SWG 121 and Lechien, were used throughout the experiments. These strains had been originally isolated from gas gangrene cases. They were stocked at room temperature, in brain Vf broth sealed in vacuo.

Liquid media. Trypticase Soy Broth (TSB; BBL) and Vf broth (9) were used in 6- or 25-ml volumes in small or standard Hall tubes.

Solid media. TSB and Vf broth were supplemented with 1% agar, 20% (w/v) ferric ammonium sulfate solution to 1% volume, 20% (w/v) sodium sulfate solution to 1% volume, and finally 0.1% (w/v) potassium nitrate. The latter was added to reduce gas production, whereas the former two salts solutions contributed to the formation of black colonies and were added to media prior to seeding. All of the media were boiled for 20 min prior to use.

Acridine derivative. As mutagenic agent, EU or 3,6-diamino-10-methylacridinium chloride (7), from British Drug Houses, Toronto, was used. EU was dissolved in distilled water to 1:1,000, sterilized by filtration and stored in the dark at room temperature. In solid media, EU was used at final concentrations of 1:10, 1:2 × 10^3, and 1:3 × 10^3. In liquid media, EU was added to the media prior to seeding, at final concentrations of 1 to 10 μg/ml.

General methods. In preliminary experiments, the gradient technique of Szymbalski and Bryson (11) was adapted to our needs to isolate resistant mutants of the anaerobic bacteria under study. Gradient plates were prepared by pouring a first layer of medium in a tilted petri dish; after allowing this layer to solidify, a second layer containing a known concentration of EU was subsequently poured on top of the first layer while the plate was on a level plane. EU diffuses in the bottom layer to form a gradient of concentrations from practically nil to the maximal concentration used. The surface of the plate was then seeded with 0.5 ml of a fourfold dilution of a 6-hr-old culture, thus providing enough cells to obtain confluent growth on control (EU-free) plates. The seeded plates were incubated 24 hr at 37 C in a nitrogen atmosphere.

After this first incubation, a dense area of growth to the limit of the tolerated EU concentration was noted; beyond this area, a few isolated colonies were found. These colonies probably represent clones from
resistant mutants; they were then streaked toward areas of still higher EU concentrations. After a second incubation similar to the first, the distal part of the bacterial growth (highly resistant mutants) was picked up and transferred into liquid media without EU.

In a second series of experiments, *C. perfringens* was incubated during 24 hr at 37 C in liquid media containing 1 to 10 μg of EU per ml. After this incubation period, samples were transferred continually in media without EU.

The cultures obtained with the help of both methods were then serially diluted in gelatin-saline; 0.1 ml of the third, fourth, and fifth dilutions was immediately incorporated in blood agar and was incubated under nitrogen at 37 C. After incubation, the blood-agar plates showed numerous typical colonies surrounded by two zones of hemolysis; the large outer zone was plate, 20\% (w/v) solution, and the small and clear zone by α-toxin. In addition, several colonies showed only one zone of hemolysis due to α-toxin, the hemolytic zone due to α-toxin having almost entirely disappeared (Fig. 1). These colonies were then serially transferred to check their purity.

In all cases, the control cultures (without EU) always showed colonies surrounded by two typical zones of hemolysis. Frequency. From the blood-agar plates seeded with the EU-treated cultures, a count was made of the total number of all the hemolytic colonies. The relation (colony outside α-toxin hemolytic zone) × 100\% (total number of colonies) was used as an indicator of the frequency of abnormal colonies after a given time of incubation.

**Count of viable bacteria.** For viable counts, 48-ml volumes of TSB were supplemented with 1\% agar and 0.1\% potassium nitrate. The culture to be counted was decimally diluted in gelatin-saline. A 0.1-ml amount of the fourth, fifth, sixth, and seventh dilutions was incorporated in 48 ml of media to which 1 ml of ammonium ferric nitrate, 20\% (w/v), solution and 1 ml of sodium sulfite, 20\% (w/v), solution were added. The entire mixture was poured into the top of a petri dish, and the bottom was delicately placed on the surface of the still molten medium, care being taken not to incorporate air bubbles (8). After solidification at room temperature, the petri dishes were incubated at 37 C for 24 hr in air. After incubation, all the black colonies, even those growing between the vertical sides of the petri dish, were counted.

**Growth curves.** Two 1-liter Erlenmeyer flasks containing 750 ml of liquid media were seeded, one with 75 ml of standard suspension (standardized to 20\% transmission at 450 m\(\mu\) with a Coleman Junior Spectrophotometer) of a mutant strain, and the other with the wild-type strain. Incubation was at 37 C in a water bath; at 0, 1, 2, 3, 4, 5, 6, and 7 hr, a 10-ml sample was taken from each Erlenmeyer flask for pH determination, optical density measurements, and viable count and lecithinase activity determinations.

**Lecithinase activity.** The lecithotellin technique of van Heyningen (2) was used to determine the lecithinase activity of supernatant fluids from *C. perfringens* treated with EU or untreated. The readings were taken on a Coleman Junior Spectrophotometer at 650 m\(\mu\). The θ-toxin was determined with sheep red blood cell suspended in phosphate buffer (3).

**Sugar fermentation tests.** For all strains used in this work, the capacity to attack glucose, sucrose, galactose, maltose, levulose, mannitol, and lactose were determined with by use of the techniques described by Prévot (8).

**Physiological properties.** Again, for all strains used here, the ability to coagulate milk, to liquefy gelatin, and to attack coagulated serum and egg white was also determined by Prévot's techniques (8), as was terminal redox coefficient (rH).

**Immunoelectrophoresis.** This study was carried out in a LKB apparatus, by use of standard microscope glass slides coated with 0.45\% (w/v) Igonor No. 2 (Oxoid) in borate buffer, pH 7.1. The culture filtrates were introduced into the inlays, and paper wicks were used to connect the ends of the gels to the buffer in the electrode chambers; 100 v and 5 ma were usually used. After 4 hr, commercial gas gangrene polyvalent antitoxin (Lederle Laboratories, Pearl River, N.Y.) was added to the longitudinal ditches of the gels, which were then usually incubated for 24 hr at 37 C.

**Animal tests.** Toxicity of the cultures was tested in 17- to 20-g male and female mice by injection into the tail vein of 0.5 ml of 6-hr culture filtrates.

To measure and compare the virulence, 0.5 ml of graded dilutions of standard suspensions of washed bacilli in gelatin-saline was injected into the thigh muscle of 6- to 7-day-old chicks. Each dilution (1:2, 1:4, 1:8, 1:16, and 1:32) was administered to a group

![Fig. 1. Blood-agar showing normal and abnormal colonies of *Clostridium perfringens*. The large and dense zones of hemolysis are due to θ-toxin; small and clear zones are due to α-toxin. Arrows indicate abnormal colonies surrounded with almost normal hemolysis due to θ-toxin, whereas hemolysis due to α-toxin is almost missing.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
of 20 chicks, while the viable bacilli were counted simultaneously.

Normal and irradiated guinea pigs, weighing 350 to 400 g, were injected in the thigh muscle with 1 ml of graded dilutions of standard suspensions of washed bacilli; the guinea pigs were irradiated in a Gammacell 220 (Atomic Energy of Canada Ltd, Ottawa) and received a whole-body single dose of γ-rays (290 r), sufficient to destroy the immunocompetent cells for a period of 5 to 10 days.

RESULTS AND DISCUSSION

The gradient plate technique gave us respectively 10 and 5 mutant strains from C. perfringens strains SWG 121 and Lechien. In EU liquid media, the Lechien strain produced four mutant strains. These mutants were all characterized by the loss of the capacity to synthesize lethal amounts of lecithinase when grown in TSB as well as in VF broth. For the sake of clarity, we present here only the results obtained with M9 and M26 mutant strains, obtained, respectively, from Lechien and SWG 121 strains.

The morphology of the mutant bacilli was the same as that of the wild-type bacilli. However, in the EU media, long filamentous shapes appear, but they segmented when EU was removed. The capsule was still present around the mutant bacilli. Wild-type strain SWG 121 is normally so mucoid that treatment with the Waring Blendor is necessary to obtain normal fluid culture (1); all mutant strains derived from SWG 121 were nonmucoid, particularly M26. This loss of viscosity means the loss of a certain polymerase responsible for production of the slime layer in SWG 121.

The mutant strains M9 and M26 did not coagulate milk, whereas wild-type strains did so easily and rapidly. The loss of the enzyme responsible for this action is permanent, as checked by repeated tests.

Terminal rH determinations indicated that mutant strains kept the same reducing capacity as the wild types.

Among the sugar fermentation tests, the M26 mutant did not hydrolyze sucrose, whereas M9 mutant and wild-type cultures fermented this sugar; all other sugars were fermented by both.
Thus, it would appear that, in the SWG 121 mucoid strain, the saccharolytic enzyme which hydrolyzes sucrose plays a role in the synthesis of the slime layer, because its M26 mutant is non-mucoid and cannot ferment sucrose.

In vitro hemolytic tests showed that mutants M9 and M26 grown in Vf broth or in TSB media synthesize very low amounts of α-toxin. Finally, the lecithinase determinations, by use of lecithovitellin, made in parallel with the growth curves, showed that the culture filtrates from mutants contained at least one-tenth as much α-toxin as the wild-type culture filtrates, whereas θ-toxin remained the same.

The immunoelectrophoretic patterns (10) confirm the above findings (Fig. 2).

The growth curves of wild-type SWG 121 and Lechien strains and of mutant strains M26 and M9 grown in TSB media showed that, after 6 hr of incubation, the numbers of viable cells were quite different. The SWG 121 culture in TSB media contained about 100 $\times$ 10$^6$ living cells per ml, and the M26 culture contained more than 450 $\times$ 10$^6$ living cells (Fig. 3). This high rate of multiplication must be borne in mind when discussing virulence. The lecithinase determinations done in parallel with the growth curves clearly showed that cultures of the wild-type strains Lechien and SWG 121, in TSB media, contained very high titers of α-toxin, whereas cultures of the mutant strains M9 and M26 (Fig. 3 and 4) contained negligible amounts of α-toxin. Moreover, when Lechien and M9 strains were grown in Vf broth (Fig. 5), the differences in α-toxin content appeared to be greater. The pH curves seem to be the same in the wild-type and mutant cultures.

In all cases, maximal α-toxin synthesis was reached between 5 and 6 hr. Vf broth appeared to be more suitable because of the higher viability and the higher α-toxin rates; this fact had been established previously at least for C. tetani (9).

Independently of the media and the strains

**Fig. 4. Determinations of viable count, lecithinase, and pH of wild-type Lechien and mutant M9 grown in TSB media.**

**Fig. 5. Determinations of viable count, lecithinase, and pH of wild-type Lechien and mutant M9 grown in Vf broth.**
TABLE 1. Virulence of Clostridium perfringens for normal and irradiated\(^a\) guinea pigs weighing 350 to 400 g

<table>
<thead>
<tr>
<th>Injected doses</th>
<th>Normal guinea pigs(^b)</th>
<th>Irradiated guinea pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type SWG 121</td>
<td>Mutant M 26</td>
</tr>
<tr>
<td>85%(^c) Dilution 1:10</td>
<td>Abdominal gg(^d)</td>
<td>No symptoms</td>
</tr>
<tr>
<td>Dilution 1:15</td>
<td>Swelling</td>
<td>No symptoms</td>
</tr>
<tr>
<td>Dilution 1:50</td>
<td>No symptoms</td>
<td>No symptoms</td>
</tr>
</tbody>
</table>

\(^a\) Guinea pigs received whole-body irradiation in a Gammacell 220.
\(^b\) In normal and irradiated animals, each dilution of washed bacilli was injected into the thigh muscle of two guinea pigs.
\(^c\) Percentage of transmission of the washed bacilli suspension at 450 m\(\mu\).
\(^d\) The symbol gg refers to gas gangrene.

TABLE 2. Virulence of Clostridium perfringens for 6- to 7-day-old chicks

<table>
<thead>
<tr>
<th>Injected viable bacteria</th>
<th>Deaths/no. injected(^e)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type SWG 121</td>
<td>Mutant M 26</td>
<td>Wild-type SWG 121</td>
<td>Mutant M 26</td>
</tr>
<tr>
<td>1,100 (\times) 10(^4)</td>
<td>20/20</td>
<td>0/20</td>
<td>20/20</td>
<td>0/20</td>
</tr>
<tr>
<td>155 (\times) 10(^4)</td>
<td>20/20</td>
<td>0/20</td>
<td>20/20</td>
<td>0/20</td>
</tr>
<tr>
<td>73 (\times) 10(^4)</td>
<td>20/20</td>
<td>0/20</td>
<td>20/20</td>
<td>0/20</td>
</tr>
<tr>
<td>38 (\times) 10(^4)</td>
<td>2/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>18 (\times) 10(^4)</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>8 (\times) 10(^4)</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

\(^a\) Doses of 0.5 ml of washed bacilli suspensions were injected into the thigh muscle.

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**Fig. 6.** Percentage of viable mutants present in 6-hr-old cultures of Lechien strain containing 1 to 10 \(\mu\)g of EU/ml (maximum obtained with 3 \(\mu\)g/ml). Used, the numerous growth curves and \(\alpha\)-toxin determinations prompted us to look for evidence concerning differences in pathogenicity of *C. perfringens*. Intravenous injections of culture filtrates to groups of four mice indicate that the mutants derived from fully toxic parent strains were not toxic at all.

To distinguish more precisely between toxicity and virulence, we injected normal and irradiated guinea pigs. These experiments indicated that, even with the extremely susceptible irradiated guinea pigs, the M26 mutant had lost its capacity to invade and produce gas gangrene symptoms, despite its high rate of multiplication in vitro. The irradiated and normal guinea pigs injected with equivalent numbers of wild-type bacilli died in 24 to 48 hr from characteristic gas gangrene (Table 1). The M9 and M26 mutants injected into 6- to 7-day-old chicks never killed. As many as 1.1 \(\times\) 10\(^4\) living mutant bacteria could not kill one chick out of many groups of 20 which were injected; moreover, no symptoms could be noted. On the other hand, 50 \(\times\) 10\(^4\) to 60 \(\times\) 10\(^4\) living wild-type bacteria (SWG 121 strain) killed about half of the injected chicks (Table 2).

All these facts suggest that EU-treated *C. perfringens* lost its capacity to synthesize \(\alpha\)-toxin (lecithinase C) and to invade the animal body. Because of the rather high frequency of this occurrence (more than 8%; Fig. 6) and of the fact that EU-treatment always gives avirulent bacilli, it is not unthinkable that an episome or a bacteriophage is in fact responsible for the discharge of \(\alpha\)-toxin. Work on that hypothesis is in progress.
ACKNOWLEDGMENTS

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LITERATURE CITED