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Stable L-Forms of *Clostridium perfringens*: Growth, Toxin Production, and Pathogenicity

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Growth and toxin production of stable L-forms of *Clostridium perfringens* grown in a mini-fermentor were monitored. A gradual but steady increment in viable count occurred over a 7-h period, followed by death. The peak of viability preceded the optical density peak by 3 h. Theta, alpha, kappa, and lambda toxins were measured, with theta toxin appearing first in the culture supernate. Growth of the parent bacillus form of *C. perfringens* was compared under similar conditions. Toxin levels achieved by the bacillus culture exceeded those of the L-form culture four- to eightfold; however, based upon viable count, the L-form organism produced 8 to 16 times as much toxin as did the bacillus. The amounts of extracellular toxin produced by both forms were similar when related to cell protein rather than cell number. Guinea pig inoculation showed that the L-form of *C. perfringens* did not produce gas gangrene, although it was not entirely without effect. Both guinea pig and human sera were inhibitory to these L-forms, a fact attributable to a heat-labile component in the sera, most likely complement.

There is no consensus of opinion concerning the pathogenicity of bacterial L-forms (2, 5, 8, 12), and the evidence presented in two papers (J. Bittner and V. Vionesco, Abstr. IX Int. Congr. Microbiol., p. 353-354, 1966; 7) dealing with the pathogenicity of L-forms of *Clostridium perfringens* is contradictory. We recently described the induction of a stable L-form of *C. perfringens* (11) and presented some preliminary data on the nature of this organism. In this paper the growth, toxin production, and pathogenicity of such L-forms are described.

MATERIALS AND METHODS

Cultures and media. The cultures and media used in these experiments were described previously (11). The stable L-form of *C. perfringens* (originally produced by penicillin treatment of the Lechien strain of *C. perfringens*) was preserved in semisolid TSA medium (brain heart infusion [BHI] broth [Difco Laboratories], 0.1% sodium thioglycolate [wt/vol], 10% sucrose [wt/vol], and 0.25% agar [wt/vol]) at 4°C or in the lyophilized state. Subsequent subcultures required no penicillin, and routine culturing of the L-forms was performed in fluid TSA medium containing 0.05% agar. A second stable L-form culture of this strain was maintained in the absence of sucrose and was utilized in the pathogenicity experiments. TSA medium prepared without agar was referred to as TS broth. Unless otherwise stated, all incubation was done at 37°C under the anaerobic conditions described previously (11).

Viable counting of L-forms and bacteria. The procedure for performing viable counts of L-forms of *C. perfringens* has been described (11). Essentially, 1 ml of serial 10-fold dilutions of L-form cultures was inoculated respectively into 10 ml of boiled, semisolid TSA medium that had been cooled to 42°C. Duplicate tubes for each dilution were incubated, and the colonies were counted after 18 h. The viable count was expressed as colony-forming units per milliliter.

Viable counting of bacteria (parent bacillus culture of *C. perfringens*, designated as strain 13 in our collection) was performed by streaking onto blood agar plates duplicate samples of serial 10-fold dilutions of culture and incubating them anaerobically for 18 h.

Growth experiments. TSA medium (100 ml) was inoculated with 10 ml of an 18-h L-form culture and incubated for 18 h. This culture was then centrifuged at 6,000 × *g* for 10 min, and the sedimented cells were suspended in 15 ml of TS broth. The L-forms were centrifuged, resuspended in 10 ml of TS broth and inoculated by syringe into a 1-liter fermentation vessel (Miniferm, Microbial Fermentor model M-1000, Fermentation Design, Inc., Allentown, Pa.) containing 500 ml of TS broth at 37°C. Nitrogen gas, passed through a sterile glass wool filter, was constantly bubbled through the medium, and a magnetic stirrer mixed the culture. At zero time and at hourly intervals, a 7-ml sample was removed from the fermentor for determination of optical density (OD) at 660 nm, viable count, pH, and toxin titer. The sample was centrifuged, and the supernatant fluid was used for the pH and toxin determinations.

A similar experiment was done with the bacillus culture of *C. perfringens*, by using the same medium

but with a starting inoculum of 1.5 ml of washed bacilli to provide an initial OD of approximately 0.05.

Toxin assays. Alpha toxin (lecithinase C) was measured by the egg yolk method described by MacFarlane et al. (9). Serial doubling dilutions of culture supernates were made in 0.5-ml volumes of 0.85% saline containing 0.5 mg of calcium chloride per ml. To each was added 0.5 ml of egg yolk solution. After incubation in a water bath for 30 min, the dilution of the last tube showing opacity was considered the titer, and the reciprocal of this dilution represented the number of units of toxin activity.

The hemolysin, theta toxin, was assayed as follows. Serial doubling dilutions of the culture supernatant fluid were made in 0.5-ml volumes of 0.85% saline containing 4% sodium citrate (to inhibit hemolytic activity of alpha toxin) and 0.1% sodium thioglycolate. To the dilutions was added 0.5 ml of 0.5% washed sheep erythrocytes (BBL, Becton, Dickinson & Co.). These tubes were shaken, incubated in a water bath, and read for complete hemolysis after 30 min. The units of activity were expressed as for alpha toxin.

Kappa toxin, or collagenase, was crudely measured as follows. Serial doubling dilutions of the culture supernatant fluid were made in 3-ml volumes of distilled water. From each dilution, 1 ml was added to 5 mg of collagen (Nutritional Biochemicals Corp., Cleveland, Ohio) in test tubes. These were incubated for 24 h and read for dissolution of the collagen. Activity was expressed as for the above toxins.

Lambda toxin (gelatinase) was titrated by adding 1 ml of the dilutions described above under kappa toxin to tubes containing 1 ml of gelatin (1%, wt/vol) at 37°C. After 24 h at 37°C, the tubes were chilled in an ice bath and read for liquefaction. The highest dilution of toxin that demonstrated liquefaction determined the titer of gelatinase.

Relationship of total protein, cell number, viable count, and toxin production. L-form and bacillus cultures of *C. perfringens* were grown in 10 ml of TS medium and BHI broth, respectively. After 6 h of growth, viable counting and direct cell counting (Petroff-Hausser bacteria counter) were performed on each culture. The remaining volumes of the cultures were centrifuged, and the supernatant fluids were assayed for theta toxin, which was used as an index of toxin production. The pellets were washed three times by alternate suspension in cold 0.85% saline and centrifugation. The final suspension in 5 ml of saline was used for protein determinations.

Protein was extracted from L-form and bacillus cultures by precipitation with an equal volume of cold 10% trichloroacetic acid. The precipitate was washed twice with cold 5% trichloroacetic acid, and then the protein was determined for each sample according to the method of Oyama and Eagle (13).

Isotope incorporation by the L-form and bacillary form of *C. perfringens*. A comparison of isotope incorporation (^{14}C-labeled amino acids, New England Nuclear Corp., Montreal, Canada), toxin production, and viability was made between the L-form and bacillus culture of *C. perfringens*. Seven 10-ml volumes of TS broth and of BHI broth containing 0.3 μCi of ^{14}C-labeled amino acids per milliliter were inoculated with 1 ml of washed L-forms and bacilli, respectively. At zero time and at 1-h intervals, one tube of each culture was removed from a 37°C water bath, and a portion from each tube was removed for viable count. Cold 10% trichloroacetic acid (3 ml) was added to 3 ml of culture in an ice bath and, after 15 min, the trichloroacetic acid precipitate was collected on a 2.4-cm Whatman glass fiber filter paper and washed five times with cold 5% trichloroacetic acid and once with ether. The filters were dried, placed in 15-ml volumes of scintillation fluid {2,5-diphenyloxazole, 4 g/liter of toluene + *p*-bis-[2]-(5-phenyloxazolyl)-benzene, 0.05 g/liter of toluene; New England Nuclear Corp.}, and counted for two 1-min intervals in a Nuclear-Chicago Isocap/300 liquid scintillation counter.

Classical toxin typing of the Lechien bacillus and L-form cultures. To confirm that the bacterial and L-form cultures under investigation behaved as classical toxin type A organisms, each was typed by toxin neutralization as described by Dowell and Hawkins (3) and by Burroughs Wellcome and Co., London. Both intraperitoneal and intravenous inoculation routes were used, respectively, with pairs of mice. Wellcome Clostridial Diagnostic Serum (*C. welchii*) was used for neutralization. L-forms were grown in TSA medium rather than cooked-meat medium for the above tests.

Animal experimentation. Three different preparations of microorganisms were used for pathogenicity studies: strain 13 parent bacillus culture grown for 4 h in BHI broth; stable L-form of strain 13 grown for 5 h in TS broth containing 10% sucrose; and stable L-form of strain 13 (not requiring osmotic stabilizer) grown for 5 h in BHI broth containing 0.1% sodium thioglycolate but no sucrose. These cultures were washed by two cycles of centrifugation at 6,000 $\times$ *g* and suspension in fresh media. The final suspensions were made as follows. The bacillus and L-form cultures grown without sucrose were suspended in 0.85% saline containing 10 μg of adrenalin per ml (1; epinephrine hydrochloride solution, C.S.D. 1:1,000, Parke, Davis & Co., Ltd., Brockville, Ontario); L-forms grown in TS broth were resuspended in sucrose/saline solution (10%/0.85%) containing 10 μg of adrenalin per ml.

Viable counts were performed on each preparation before injecting 0.5 ml intramuscularly into the thigh of 300- to 400-g guinea pigs. Three guinea pigs were inoculated with each preparation, respectively. As controls, two animals each were inoculated with 0.5 ml of 10% sucrose solution, 0.85% saline containing 10 μg of adrenalin per ml, and 10% sucrose solution containing 10 μg of adrenalin per ml, respectively.

Effect of serum on L-forms of *C. perfringens*. A 4-h, 10-ml culture of L-forms was centrifuged at 6,000 $\times$ *g*, and the pellet was suspended in 5 ml of TS broth. This was distributed in 1-ml volumes, to which was added 1 ml of the following sera: fresh guinea pig serum, heat-inactivated guinea pig serum (56°C for 30 min), single human serum, and human sera pooled from three persons. A control

tube received 1 ml of TSA broth rather than serum. After 30 min in a 37°C water bath, these cultures were assayed for viability.

RESULTS

Growth experiments. The growth characteristics of stable L-forms of *C. perfringens* when cultured in a small fermentor containing TSA medium are shown in Fig. 1. There was a gradual but steady increment in viable count over a 7-h period, after which death of the culture appeared to be logarithmic. The viability peak (7 h) preceded the optical density peak by 3 h. A lag period of 4 h was experienced when OD was the parameter of growth. A gradual fall in pH was noted from the second hour of the experiment to its conclusion at 24 h.

Toxin production by this culture is presented in Fig. 2. Theta toxin was first detected at 1 h and reached a maximum of 64 U by 5 h. Alpha toxin was not detectable until 4 h and reached a plateau of 32 U by 7 h. Collagenase and gelatinase titers were much lower than those of alpha and theta toxins, but this may only be a reflection of the assay system. The results presented in these figures are representative of three identical experiments.

Data obtained from the growth study of the bacillary form of *C. perfringens* executed in like manner to the L-form experiment are presented in Fig. 3. The viable count preceded but paralleled the OD readings over a 4- to 5-h growth period. There was no marked drop in OD or viable count after the stationary phase of growth had been achieved. The maximum OD and viable count of the bacillus culture exceeded that of the L-form culture by 3 and 64

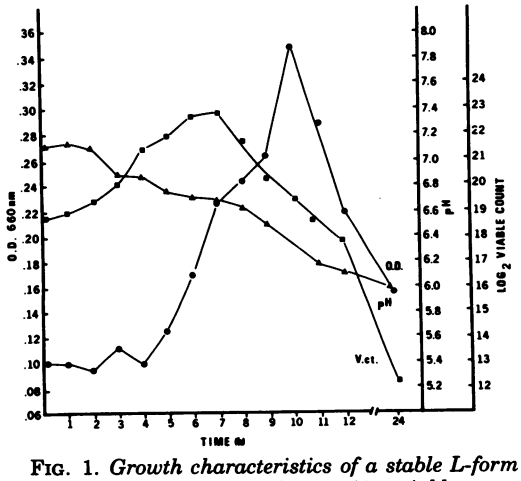

FIG. 1. *Growth characteristics of a stable L-form of C. perfringens. Optical density (●), viable count (■), and pH (▲).*

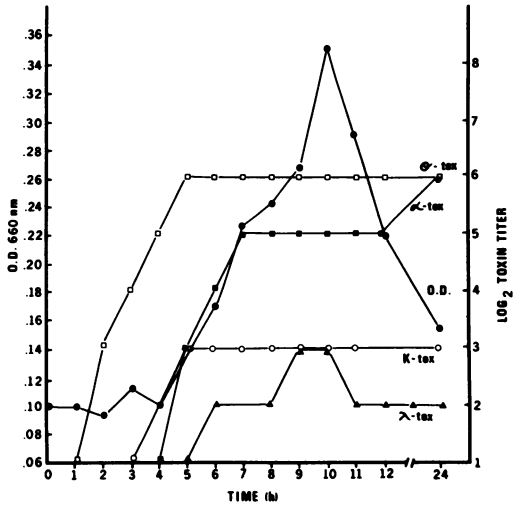

FIG. 2. *Toxin production by a stable L-form of C. perfringens. Optical density (●), theta toxin (□), kappa toxin (○), alpha toxin (■), and lambda toxin (▲).*

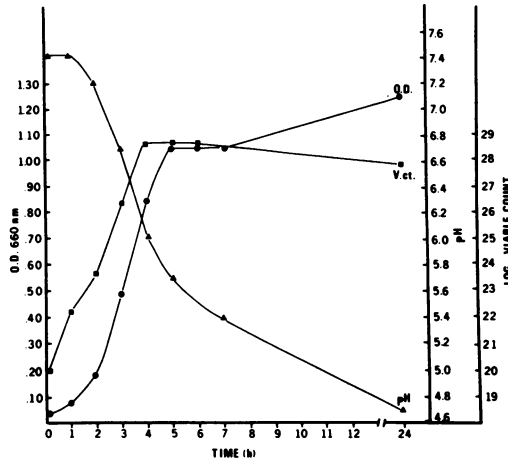

FIG. 3. *Growth characteristics of the parental bacillus form of C. perfringens. Optical density (●), viable count (■), and pH (▲).*

times, respectively. Figure 4 shows toxin production by the bacillus culture. Theta toxin was again the first to be detected at 1 h, whereas the other toxins were detectable by 2 h. The maximum titers of all toxins were achieved at the peak of viable growth, i.e., at 4 h. All toxin titers of the bacillus culture exceeded those of the L-form culture by a factor of four to eight.

The ratio of toxin titer produced by the L-form and bacillus cultures is shown in Table 1. Based upon the maximum titer produced by each culture, the L-form culture produced one-fourth to one-eighth the amount of toxin pro-

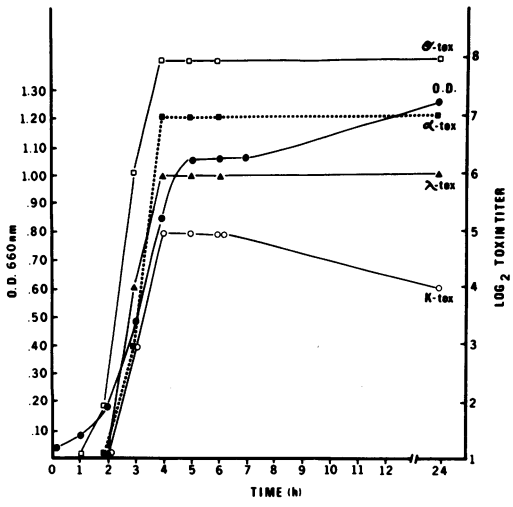

FIG. 4. *Toxin production by the parental bacillus form of C. perfringens. Optical density (●), theta toxin (□), alpha toxin (■), lambda toxin (▲), and kappa toxin (○).*

TABLE 1. *Ratio of toxin produced by the L-form and the bacillary form of C. perfringens grown in a fermentor*

Toxin	Ratio based on maximum titer/ culture	Ratio based on maximum titer/ viable organism
Theta	1:4	16:1
Alpha	1:4	16:1
Lambda	1:8	8:1
Kappa	1:4	16:1

duced by the bacillus culture. However, when the ratios of toxin produced per viable organism were compared, the L-form appeared to produce 8 to 16 times more toxin per viable cell than did the bacillus.

Relationship of total protein, cell number, viable count, and toxin production. Stationary L-form and bacillary form cultures of *C. perfringens* were grown in 10 ml of TS and BHI medium, respectively. These cultures were analyzed for viable count, cell number, protein, and theta toxin (Table 2). The number of L-forms (spheroplasts) detected in a bacterial counting chamber exceeded the viable count of the culture by a factor of 3.3. This count does not take into account the presence of any granules (should they be of significance) since they are too small to be counted reliably by eye. The ratio of counted bacilli to viable bacilli was 2.2. The amount of theta toxin produced by the L-form and bacillus culture in this small-volume stationary culture was greater than that produced in the fermentor cultures described above.

When the amount of toxin and protein per viable cell was examined, the ratios of each produced by the L-form compared with the bacillus were very similar, i.e., 7.1 and 6.5, respectively. Thus, although the amount of toxin produced per viable L-form considerably exceeded that produced per viable bacillus, the amount of protein in the L-form also exceeded that of the bacillus by approximately the same proportion. Therefore, toxin production should be related to cell mass and not to cell number.

Isotope incorporation by the L-form and bacillary form of *C. perfringens*. This experiment was performed to compare the rate of protein synthesis in the L-form and bacillus culture, and to relate this to toxin production and viability. The rate of ^{14}C-labeled amino acid incorporation was about the same for both the L-form and bacillary form of *C. perfringens*; however, the amount of incorporation per viable form was much greater than that of the bacillus. The amounts of theta toxin produced by both forms were the same in this experiment (256 U). An analysis of isotope incorporation, viable count, and toxin production at 4 and 6 h is recorded in Table 3. The amount of isotope incorporation was proportional to the amount of toxin production in each case, again indicating that cell mass, and not cell number, is the critical factor in determining toxin production.

Animal experimentation. Both the bacillus and L-form cultures behaved as typical toxin type A strains when injected intraperitoneally or intravenously into mice. Mice injected intraperitoneally with 24-h cultures died in 24 h unless the culture was previously neutralized with type A antitoxin. Intravenous injection resulted in death within 10 to 15 min.

Guinea pigs were chosen as a model for the classical gas gangrene experiments. In each animal, typical gas gangrene developed when

TABLE 2. *Comparison of direct count, viable count, toxin production, and protein content of the L-form and bacillary form of C. perfringens*

Measurement	Type of culture	
	L-form	Bacillus
Direct count	9.2×10^7/ml	8.6×10^8/ml
Viable count	2.75×10^7/ml	3.9×10^8/ml
Toxin titer	512 U	1,024 U
Protein	640 μg/ml	1,400 μg/ml
Toxin/viable unit	18.6×10^{-6} U	2.6×10^{-6} U
Protein/viable unit	2.3×10^{-5} μg	3.6×10^{-6} μg
Toxin ratio, L-form/bacillus	7.1	
Protein ratio, L-form/bacillus	6.5	

TABLE 3. *Analysis of isotope incorporation, viable count, and toxin production by the L-form and bacillary form of C. perfringens*

Measurement	Type of culture			
	4 h		6 h	
	L-form	Bacillus	L-form	Bacillus
Viable count	2.45×10^7/ml	6.9×10^7/ml	4.05×10^7/ml	6.9×10^8/ml
Toxin titer	64 U	64 U	256 U	256 U
256 U				
Ratio of toxin, L-form/bacillus	2.8		17	
cpm	21,000	18,000	49,000	62,000
cpm/viable	8.6×10^{-4}	2.6×10^{-4}	1.2×10^{-3}	9×10^{-5}
Ratio of cpm, L-form/bacillus	3.3		13	

the animals were injected with the bacillus culture of *C. perfringens*, and death occurred shortly after 24 h. The viable count of the inoculum was 3.6×10^8 bacteria/ml. There was extensive digestion of muscle tissue, with almost complete dissection of the inoculated muscle. Tissue damage extended into the abdomen and the other hind limb. *C. perfringens* was isolated in pure culture from these locations, with a decreasing number of organisms being found in the upper abdomen and pleural cavities.

When L-form cultures were injected into guinea pigs, there was no similar reaction. The viable counts of the inocula were 4.3×10^7 organisms/ml for the L-forms in 10% sucrose and 2.9×10^8 organisms/ml for the L-forms without sucrose as stabilizer. In each group of animals, there was a notable swelling of the inoculated limb by 18 h, and the animals demonstrated reduced activity. The limb was obviously favored as shown by limping. Some animals were sacrificed by 48 h (none spontaneously died or appeared sick enough to die). Upon autopsy, the tissues looked healthy and intact without signs of gas gangrene. We were unable to culture L-forms from such tissue. Animals that were allowed to survive for several days appeared to regain much use of the limb. Control animals injected with saline + adrenalin, sucrose + adrenalin, or sucrose alone demonstrated no reaction to the inocula. It was also demonstrated that 10 μg of adrenalin per ml had no adverse effect against growing L-forms in test tube experiments.

When L-forms were heavily irradiated with ultraviolet light before inoculation, no reaction occurred in the limb. The supernatant fluid of the L-form culture also produced no observable reaction in the test animal.

Effect of serum on L-forms of *C. perfringens*. A considerable decrease in viability of L-form cultures was noted after treatment for 30 min with fresh guinea pig sera and human sera (Table 4). Heated guinea pig serum (56°C for 30 min) did not kill the L-forms, but allowed growth to occur. There was no adverse effect upon bacillus viability when tested as above.

TABLE 4. *Effect of guinea pig and human sera on L-form viability*

Treatment (37°C, 30 min)	Viability (CFU[a]/ml)	% Change
Control, TSA broth	1.3×10^7	
Fresh guinea pig serum	5×10^6	−62
Heated guinea pig serum	2.4×10^7	+185
Single human serum	3.9×10^6	−70
Pooled human sera	3×10^6	−77

[a] CFU, Colony forming units.

DISCUSSION

There is little doubt that L-forms of *C. perfringens* produce toxins, and many of the means usually used to measure bacterial growth may also be used for the study of L-forms. The purpose of using a fermentor in these experiments was to reduce clumping of L-forms and to reduce the formation of syncytial masses that often occur in stationary cultures, although better growth and toxin production may occur in the latter. The OD achieved by the L-form is about one-third that observed for the bacillus culture and, from our data, the generation time of *C. perfringens* L-forms is 66 min which is about triple that of the bacillus. Although we have not specifically studied the mode of division in these L-forms, it would appear that many of the spheroplast forms demonstrate budding. The maximum viable count of the L-form culture is greatly exceeded by that of the bacillus culture, and the total toxin production of the latter also exceeds the former by several fold. However, on a per-viable-unit basis, the L-form organism would seem to produce more toxin than the bacillus. Such was the observation of Scheibel and Assandri (14) when L-forms of *C. tetani* were examined. Based upon cellular protein content and isotope incorporation, the amount of toxin produced by

both cultures of *C. perfringens* is much more related to protein content than to cell number; thus the large size of the L-form spheroplasts accounts for the higher toxin level per cell.

The measurements of toxin in these experiments are only meant to provide crude estimations of toxin levels. We chose not to use 50% end points for the toxin titrations since it has been our experience that little more information is established by this method as compared with the complete end-point titration. We have referred to gelatinase activity as lambda toxin, although lambda toxin is apparently not produced by classical type A strains of *C. perfringens* (15). The gelatinase activity is more likely a reflection of kappa toxin activity that also attacks gelatin. The separation of toxins by time sequence is interesting in the L-form culture, and it is conceivable that by terminating growth of an L-form culture of *C. perfringens* at 3 to 4 h, theta toxin might be selectively obtained free from most other toxins produced by the organism.

The drop in pH of the bacillus culture grown in TS broth is much more acute than that of the L-form culture. This may be a reflection of the amount of metabolism occurring in each system, or it may suggest that sucrose is not utilized by the L-form to the same extent as it is by the bacillus. Previous work (11) has indicated that L-form growth and viability are very susceptible to pH values of 6 and below; perhaps the marked drop in viability of the L-form between 12 and 24 h is a reflection of this pH change. The bacillus viability is not affected in this manner.

Kawatomari (7) reported the growth of L-form colonies of *C. perfringens* on blood plates where some colonies showed well-defined zones of hemolysis, although hemolysis was not always manifested. In those experiments, guinea pigs and rabbits were inoculated with saline suspensions of L-forms with no reaction. No information was provided regarding use of adrenalin or $CaCl_2$ to initate gas gangrene infection in these experiments, although the author reports that the bacillus form of the *C. perfringens* strains tested did produce typical reactions in guinea pigs.

Bittner and Vionesco (Abstr. IX Int. Congr. Microbiol., p. 353–354, 1966) reported on the penicillin induction of stable L-forms of *C. perfringens* that produce titratable toxin levels and that cause typical gas gangrene in guinea pigs. In another experiment they described the induction of unstable L-forms which demonstrated diminished pathogenicity that returned to full pathogenicity upon reversion of the L-form to the bacillus form.

We found that the stable L-form produced in our laboratory did not cause gas gangrene in guinea pigs, but it was not entirely without effect on the animal. Adrenalin was used as a vasoconstrictor as described by Bullen and Cushnie (1) to initiate potential infection. The bacillus culture produced classical gas gangrene in guinea pigs, whereas the L-form had a very ill-defined effect on the animal. Autopsy revealed healthy intact muscle tissue in L-form-injected animals that appeared similar to uninoculated control limbs; however, in the living animals, swelling of the inoculated limb was apparent, and the animal favored the limb. No animals died from L-form infection, and gradual use of the limb returned. We could not culture L-forms from autopsy material. Highly ultraviolet-irradiated L-forms and various combinations of sterile reagents produced no response in control animals, suggesting that the live L-form did induce some unique response in the animal.

The fact that fresh sera from guinea pigs and humans considerably reduced the viability of the L-form may explain why the L-form has little or no pathogenicity in animals, and might also explain the inability to retrieve these organisms from the site of inoculation. Heat-inactivated sera did not kill the L-form. Therefore, it would seem that complement or some other heat-labile component of serum, and probably natural defense mechanisms of the animal, have successfully eradicated the L-form of *C. perfringens*. The effect of sera on other L-forms has been described in the literature (4, 6, 10).

Preliminary histological studies indicate an inflammatory response to L-form inoculation, however, with signs of tissue toxicity. Further investigations of tissue pathology will be made.

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