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# Biological Characterization of *Fusobacterium necrophorum* Cell Fractions in Preparation for Toxin and Immunization Studies

M. M. GARCIA, D. C. ALEXANDER, AND K. A. McKAY

*Animal Pathology Division, Agriculture Canada, Animal Diseases Research Institute, Ottawa, Ontario, K2H 8P9, Canada*[1]

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*Fusobacterium necrophorum* isolated from bovine liver abscesses was grown in bulk at 37 C for 24 h under a strict anaerobic atmosphere. Harvested washed cells were disrupted ultrasonically and fractionated by differential centrifugation into the intracellular (cytoplasm) and cell wall fractions. Both intact cells and cell fractions induced generalized cytopathic effect on primary pig kidney cultures and caused a variety of signs of illness and/or death of intraperitoneally injected mice. The intact cells, disrupted cells, and cell walls produced necrotic lesions and erythema on intradermally injected guinea pigs and rabbits, whereas the cytoplasm mainly erythema. By contrast, the used culture medium (culture filtrate) of *F. necrophorum* did not show any detectable toxicity. The toxic component of the cytoplasm appears to be associated with nondialyzable, hemolytic, high-molecular-weight proteins and its toxicity is reduced by trypsin and pronase. Heating at 60 C for 10 min decreased markedly its erythemal and cytotoxic ability, whereas the toxicity of the cell walls appeared to be only slightly affected even when heated at 100 C for 1 h. These results suggest that at least two distinct cell-bound toxic factors are present in *F. necrophorum* cells.

Although *Fusobacterium necrophorum* (*Sphaerophorus necrophorus*) (A cursory check of certain references [2, 22, 23] revealed that no less than 20 different binomials have been used at various times to refer to *S. necrophorus*. More recently, a proposal has been made to regroup members of the genus *Sphaerophorus* to either non-butyrate-producing *Bacteriodes* or butyrate-producing *Fusobacterium* [14]. Partly on this basis, *S. necrophorus* has been renamed *Fusobacterium necrophorum*.) has been widely known to cause various infections in animals and man, there is relatively scant information about the mechanism(s) by which the pathogen exerts damaging effects on the host tissues. Studies to clarify such mechanism(s) may have been hampered partly by the apparent disagreement in the results obtained by several investigators regarding the type of toxin(s) the pathogen elaborates and the location of this toxin in the bacterial cell. Furthermore, there has been an obvious lack of systematic and quantitative approach to this problem because of the difficulty, until recently, in growing the organism in bulk (9).

In 1934, Beveridge (1) reported the presence of both a soluble exotoxin in culture filtrates and a heat-stable endotoxin from strains of *Bacillus necrophorus*. Orcutt (16) induced illness and sometimes death in mice injected intraperitoneally with cultures filtrates of *B. necrophorus* isolated from bovine tissues. Comparative studies by Dack and co-workers (4) disclosed that culture filtrates from animal strains of *Bacterium necrophorum* were more toxic to laboratory animals than those derived from human strains. Exotoxins of certain *S. necrophorus* type A (hemolytic) strains were ascribed to the presence of hemolysin (6). However, Roberts (20) observed that *Fusiformis necrophorus* isolated from sheep foot abscesses elaborated a leukocidal exotoxin that had properties different from hemolysin. In contrast to the above investigators, others were unable to detect any toxin from culture filtrates of various *S. necrophorus* strains but found heat-killed, washed cells to be strongly toxic. Thus, according to them, the active factor is endotoxin (21, 22). Recently, Hofstad and Kristofferson (12) extracted what appeared to be lipopolysaccharides from three different *S. necrophorus* strains. The lipopolysaccharide preparations, however, produced inconsistent localized Schwartzman reaction in rabbits. Such apparently conflicting reports might be due to differ-

[1] Address reprint requests to Dr. R. J. Avery at this address.

ences in the bacterial strains, media, cultural conditions, animal host strains or age, and the techniques employed to demonstrate the presence of toxin(s). It should be noted that most of these studies employed either whole cell preparations or the culture filtrates. Thus, the fundamental question concerning the cellular location and properties of cell-bound toxic factors remains unresolved. A systematic approach therefore, was undertaken to locate the *F. necrophorum* cell fractions possessing toxicity and to characterize certain of the biological and biochemical properties of these fractions compared with those of intact cells. This was taken as an important first step towards the purification of its cellular toxin(s) and the development of a vaccine that would immunize cattle against infections of this economically important pathogen.

## MATERIALS AND METHODS

**Cultural.** *F. necrophorum* was isolated from bovine liver abscesses and characterized by methods described in a previous study (10). Stock cultures were stored in liquid nitrogen. The organism was recovered in deaerated fluid thioglycolate or in modified Casitone broth (9). Blood agar (5% sheep or horse blood) plates were used for retrospective checks on the purity of the culture. For large-scale cultivation of the test organism, a detailed description of the procedure is found elsewhere (9).

The cell harvest was washed twice with and resuspended in saline (0.85% NaCl). The supernatant culture medium was passed through a 0.45-$\mu$m membrane filter (Millipore Corp.), hence referred to as culture filtrate.

**Cell fractionation.** All fractionation steps were done aseptically at 0 to 5 C. Washed cells were adjusted to 15 mg (dry weight)/ml of suspension and ruptured with a MSE 100-W ultrasonic disintegrator (MSE, Cleveland, Ohio) for 18 to 20 min. Examination of the ultrasonically treated cells by phase microscopy revealed an almost complete disruption (>99%).

The fragmented cells (sonic extract) were centrifuged at 20,000 $\times$ *g* for 15 min and the resulting supernatant fluid was removed and recentrifuged at the same centrifugal force. The pellets from both centrifugations were pooled and represented the crude cell walls; the supernatant fluid constituted the intracellular fraction and was labeled cytoplasm. The crude cell walls were washed twice with 1 M NaCl followed by at least five washings of distilled water until the discrete absorbance peak at 260 nm of the ultraviolet spectrum of cell wall samples disappeared. Uniformity of the cleaned cell wall preparations from different batches was ascertained by infrared spectroscopy (3). Samples of both cytoplasm and cell walls were negatively stained with 2% phosphotungstic acid (pH 7.0) and examined for homogeneity by electron microscopy.

The cytoplasm was divided into three portions. The first was used for toxicity tests and biochemical assays. The second portion was supplemented with 0.01 M $MgCl_2$ and subjected to sequential centrifugation at 105,000 $\times$ *g* for 1, 2, and 16 h. The 1-h supernatant was further centrifuged for 2 h; likewise, the 2-h supernatant was centrifuged for 16 h. The resultant pellet from each step was resuspended in HMK buffer (0.05 M *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid, pH 7.0; 0.01 M $MgSO_4$; 0.025 M KCl) to the original volume. The third portion was precipitated with 50% ammonium sulfate at pH 6.5 and the resulting precipitate was dialyzed in three changes of saline for 48 h. The protein solution retained in the dialysis bags, now freed of ammonium salts, was referred to as intracytoplasmic extract.

Sterility of the different preparations was ascertained by culture on blood agar plates and in tubes of fluid thioglycolate under aerobic and anaerobic conditions. Samples were stored at −20 C until ready for use.

**Gel filtration.** Five-milliliter amounts of the intracytoplasmic extract were chromatographed in a Sephadex G-200 column (2.5 by 45 cm equilibrated with 0.01 M phosphate-buffered saline (pH 7.1) at a flow rate of 7 ml/h. Eight-milliliter fractions were collected and read at 280 nm in a Unicam SP 800 recording spectrophotometer (Pye Unicam Ltd, Cambridge, England). Aliquots of these fractions were sterilized by membrane filtration (0.45 $\mu$m; Millipore Corp.) and used in the intradermal test. The remaining amounts were set aside for further biochemical purification.

**Protein determination.** Protein concentrations were determined by the Folin phenol method (14) with bovine albumin fraction V as standard. Ultraviolet spectra of the cell fractions were analyzed with a Unicam SP 800 recording spectrophotometer, whereas the infrared spectrum of the dried washed cell wall preparations was determined by a Perkin-Elmer 137-B Infra Cord using KBr pellets with thickness of 100 to 200 $\mu$m.

**Mouse toxicity test.** White female Swiss albino mice (about 25 g) were injected intraperitoneally (i.p.) and intravenously (i.v.) with twofold dilutions of the various bacterial preparations. Doses for the two routes were 1.0 and 0.5 ml, respectively. Groups of six mice were used for each dilution. After a week of observation, mean lethal dose ($LD_{50}$) values were calculated according to the method of Reed and Muench (19).

**Effect of enzymes on the toxicity of cytoplasm.** The following enzymes solutions were added to various tubes containing cytoplasm: alpha-amylase in 0.01 M phosphate buffer, pH 7.0; beef pancreas ribonuclease in 0.2 M tris(hydroxymethyl)aminomethane buffer, pH 7.5; hog pancreas lipase 448 in 0.05 M citrate-phosphate buffer, pH 6.0; trypsin in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.2; and pronase in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.2. Except for pronase, which had a final concentration of 2.5 mg/ml, certain of the buffers and the concentrations used were based on Duncan and Strong (5). Pronase was obtained from Calbiochem, whereas the rest came from Nutritional Biochemical Corp. The cytoplasm-enzyme mixtures and appropri-

ate controls were incubated at 37 C for 24 h. Groups of 10 mice were injected i.p. with 1.0 ml each of the specific preparation. Observation on the mortality was done as described previously.

**Intradermal test.** English short-hair female guinea pigs, each weighing about 650 g, were used. Intradermal injection of 0.1-ml samples was done at various areas on both sides of the animals. At least 10 injections per sample were done on five guinea pigs. Skin reactions were noted at 24 h postinoculation and daily thereafter for 1 week. Certain of the guinea pigs were killed after 24 and 48 h of inoculation, and representative areas of reactions were removed for histopathological examination. This test was used (i) to evaluate the ability of intact cells and cell fractions to induce various skin reactions, (ii) to compare the effect of heat treatment, and (iii) to identify toxic fractions derived from gel filtration.

**Tissue culture assay.** Primary pig kidney cells (PKC) were grown for 3 days at 37 C in a lactalbumin hydrolyzate medium (11) modified by replacing the proteose peptone with yeast extract, the gamma-globulin-free serum supplement with fetal calf serum, and the bicarbonate buffer with 3.0 g of *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic buffer per liter. Prior to application of the various treatments, the tissue culture medium was replaced with a supplemented minimum essential maintenance medium. This maintenance medium contained: dissolved AUTO-POW MEM (Flow Laboratories, Rockville, Md.), 97.0 ml; sodium carbonate (5.6%), 0.4 ml; Neomycin (Upjohn Co. of Canada, Don Mills, Ont.), 0.1 ml of 10% solution; pyruvate (100 times concentrated), 1.0 ml; L-glutamine (200 nM), 1.0 ml; nonessential amino acids (100 times concentrated), 0.5 ml; and fetal calf serum, 10 ml. The last four constituents were obtained from Grand Island Biological Co., Grand Island, N.Y. One-milliliter amounts of *F. mecrophorum* cells and cell fractions were inoculated to each flask of 4.0 ml of tissue culture.

The effect of the various bacterial preparations on the amino acid incorporation by tissue culture cells was determined. PKC monolayers were trypsinized, washed, and suspended in minimal essential medium plus supplements at a concentration of $10^6$ cells/ml. One milliliter of the cell suspension was added to each of a set of reaction tubes, which contained 2.5 $\mu$Ci of $^{14}$C-labeled amino acid mixture (Amersham/Searle, Don Mills, Ontario) and 0.5 ml of bacterial preparation or saline. The tubes were then incubated at 37 C. Reaction was stopped immediately by the addition of 2.0 ml of cold 5% trichloroacetic acid. The precipitated proteins were pelleted by centrifugation, washed twice in saline, and solubilized with 2.0 ml of N.C.S, solubilizer (Amersham/Searle). Counting was done in a Nuclear-Chicago scintillation counter.

**Hemolytic activity.** The titration assay for hemolytic activity of Fievez (6) was essentially adopted. Briefly, twofold dilutions of the bacterial preparations were mixed with an equal volume of a 0.25% suspension of washed chicken erythocytes. Tubes containing the mixtures and appropriate controls were incubated at 37 C for 2 h. The highest dilution showing 50% lysis was regarded as the end point. Rabbit, bovine, and human erythrocytes were also employed in this test.

**Serological techniques.** New Zealand white female rabbits, each weighing about 2.3 kg, were bled prior to injection and the preimmune sera obtained were used as controls. An initial dose of 2 ml of a 1:1 antigen sodium alginate (COLAB, Weston, Ontario) mixture was injected subcutaneously into four sites on the rabbits. After a 3-day pause, increasing doses of the formolized antigen (0.05% formalin) was injected i.v. every other day for 3 weeks so that each animal received a total dose of 30 mg of protein antigen. The animals were bled a week after the last injection.

Immunodiffusion assays (17) were carried out using plates of sodium borate buffer, pH 8.0, plus 1.0% Ionagar. Incubations were done at 4, 22, and 37 C for 7, 4, and 3 days, respectively. The plates were soaked in water for 4 days, stained with 1.0% amido black in 7.0% acetic acid for 1 h, and destained for another 4 to 5 days with a 9:9:2 methanol-acetic acid-water mixture.

## RESULTS

**Relative toxicity of F. necrophorum cells and cellular fractions.** Washed intact cells, sonic extract cytoplasm, and cell walls caused death or various signs of illness in mice. The first signs of toxic response occurred within 5 h after i.p. injection of the various bacterial samples. The animals appeared weak and displayed loss of appetite, and their hair coats turned rough. As the illness progressed the animals lost most of their mobility and exhibited labored breathing, and their eyes became matted and closed. Inoculated mice that became sick but did not die within 7 days usually recovered.

Results of the various toxicity tests are summarized in Table 1. Mouse $LD_{50}$ values were expressed in terms of protein content. The sonic extract showed a slightly lower lethal dose than the intact cells and appeared to kill the mice more rapidly. Compared to the intact cells, about one-third less cytoplasmic protein was required to produce a similar toxicity level. On the other hand, the cell wall fraction appeared to be much less lethal than the cytoplasm on the basis of their protein contents. Prelimary trials on i.v. injections of the different materials revealed consistent results only with the cytoplasm ($LD_{50}$ = 0.67 mg); inconsistencies observed in some of the mice injected with the other more particulate cellular constituents (whole cells, sonic extract, and cell walls) could be due to blocking of the blood vessels.

The cytoplasm, whole cells, and sonic extract, inoculated i.p., caused generalized congestion and occasional necrotic lesions in mouse livers as well as marked paleness of the kidneys and splenomegaly. Maximum spleen enlargement, induced by the cytoplasm at sublethal dose, was attained at 4 days postinoculation, after which

TABLE 1. *Relative toxicity of F. necrophorum cells and cellular fractions*

| Material | Protein content (mg/ml) | Relative toxicity | | |
|---|---|---|---|---|
| | | Mouse $LD_{50}$[a] | Primary PKC[b] | Guinea pig skin[c] |
| Intact cells | 9.50 | 1.41 | Extensive detachment of tissue monolayer | Erythema, central area becoming necrotic |
| Sonic extract | 9.50 | 1.28 | Extensive detachment of tissue monolayer | Erythema, central area becoming necrotic |
| Cytoplasm | 4.75 | 0.84 | Widespread cell granulation and shrinkage | Mainly erythema |
| Cell walls (clean) | 3.06 | 1.72 | Cells very granular and vacuolated, less extensive detachment of monolayer | Erythema, necrotic areas occurring but less frequent |
| Culture filtrate | 5.98 | None | None | None |

[a] Values expressed in milligrams of protein, i.p. injection.
[b] Observation at 24 h postinoculation.
[c] Observation at 24 h postinoculation, intradermal injection.

the average spleen size started to approach that of the control saline group.

The effect of enzymes on the mouse toxicity of cytoplasm indicated that the toxic factor is associated with proteins (Table 2) as only trypsin and pronase, both proteolytic enzymes, reduced effectively the toxicity of the cytoplasm.

Results obtained from sequential centrifugation revealed that the toxicity of the cytoplasm resided mainly with the component sedimented at 105,000 × *g* for 1 h (Table 3).

Intradermal injections of *F. necrophorum* cells and cellular fractions produced acute inflammation of the skins of guinea pigs and rabbits. Although preliminary tests showed the rabbits to be more reactive, the guinea pigs gave more consistent reactions. Therefore, the latter were mainly used in subsequent bioassays. The intact cells and sonic extract caused both erythema and the formation of necrotic areas which began as small yellowish-white foci 24 to 28 h after intradermal injection. Microscopic examinations revealed abscesses in the deep dermis, blood vessels occluded with swollen endothelial cells, and extensive necrosis of the epidermis. Guinea pigs inoculated intradermally with cell wall preparations showed necrotic lesions, but this reaction occurred less frequently and was milder compared to that provoked by the intact cells and the sonic extract. By contrast, the cytoplasm caused mainly erythema with slight edema. After 7 days, most of the necrotic lesions were sloughed off from the skin whereas the erythema almost disappeared.

Visible cytotoxic changes were observed initially in primary PKC 5 h after inoculation of the bacterial cells and cellular fractions. This incipient effect was characterized by the general granular appearance of the tissue cell cytoplasm and rounding of the cells. At 24 h postinoculation, the cytotoxicity of the inocula became more pronounced as evidenced by extensive vacuolation, cell degeneration, and detachment

TABLE 2. *Effect of selected enzymes on the mouse lethality of F. necrophorum cytoplasm*

| Treatment[a] | Mortality (%) |
|---|---|
| Cytoplasm alone | 100.0 |
| Cytoplasm + α-amylase | 100.0 |
| Cytoplasm + ribonuclease | 91.0 |
| Cytoplasm + lipase | 100.0 |
| Cytoplasm + pronase | 27.3 |
| Cytoplasm + trypsin | 18.2 |
| Enzyme in buffer[b] | 0 |
| Buffer alone[c] | 0 |

[a] Groups of 11 mice were injected i.p. with each preparation.
[b] Applicable to all enzymes and corresponding buffers used.
[c] Applicable to all buffers used.

TABLE 3. *Bioassay of cytoplasmic components derived by sequential centrifugation at 105,000 × g*

| Fraction | Centrifugation time (h) | Survivors/total mice injected[a] | PKC[b] |
|---|---|---|---|
| Pellet | 1 | 1/10 | ++ |
| Supernatant | 1 | 10/10 | + |
| Pellet | 2 | 10/10 | ± |
| Supernatant | 2 | 10/10 | − |
| Pellet | 16 | 10/10 | − |
| Supernatant | 16 | 10/10 | − |

[a] Mice were injected i.p. at a dose of 1.0 ml.
[b] Rating of cytotoxicity of PKC: ++, moderate to severe; +, mild; ±, slight change; −, no effect.

of tissue cells from the surface of the glass. The latter response was particularly extensive in tissue cultures inoculated with the intact cells and sonic extract. An earlier effect than the above observation was revealed, however, by the radiotracer experiment (Fig. 1). A drastic reduction in amino acid incorporation occurred 2 h after the primary PKC were inoculated with the toxic bacterial samples. The intact cells suppressed $^{14}C$ uptake more severely than the cell walls or the cytoplasm. A slight lag in amino acid incorporation occurred with the control treatment presumably as a result of environmental changes attendant with the removal of the cell monolayers from culture flasks. However, the control cells appeared to have recovered by the second hour of inoculation.

Irrespective of the type of bioassay empolyed, the culture filtrate did not induce any toxic response. Furthermore, transitory signs of illness exhibited by mice injected i.p. with fourfold concentrated culture filtrate were also observed in those inoculated with the same concentration of uninoculated medium.

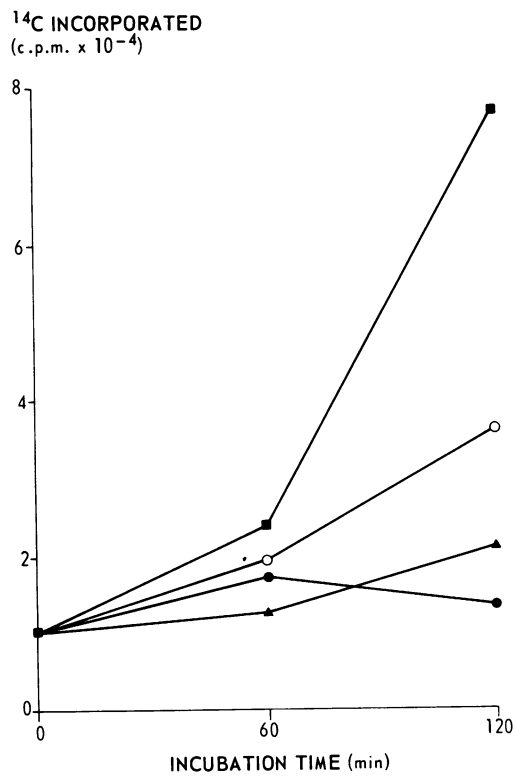


FIG. 1. *Effect of F. necrophorum cells, cytoplasm and cell walls on the $^{14}C$-labeled amino acid uptake in primary PKC. Symbols:* ●, *intact cells;* ▲, *cell walls;* ○, *cytoplasm;* ■, *saline.*

**Relative toxicity of intracytoplasmic fraction.** The intracytoplasmic extract, composed mainly of protein, possessed toxic properties similar to those of the cytoplasm. In fact, based on the protein content, the mouse $LD_{50}$ of the cytoplasmic extract (0.82 mg) approximated that of the cytoplasm (0.84 mg). Fractionation of the intracytoplasmic extract by gel filtration showed a direct relationship between absorbance of the fractions at 280 nm and their erythemal activity in guinea pig skin (Fig. 2). The fraction showing both characteristics appeared to be associated with large-molecular-weight proteins since it was eluted just after the void volume (48 ml). Subsequent fractionation of the extract with a Sepharose 6B column produced a similar result.

**Hemolytic activity.** Both cytoplasm and intracytoplasmic extract produced hemolysis of chicken erythrocytes with end points of 1:64 and 1:128, respectively. These fractions also hemolyzed rabbit, sheep, bovine, and human erythrocytes. No hemolysis was observed when the culture filtrate was used.

**Effect of heating on the relative toxicity of intact cells and cell fractions.** The various toxic cell constituents differed in sensitivity to heat treatment (Fig. 3). The erythemal activity of the cytoplasm was reduced markedly after 10 min of heating at 60 to 100 C. By contrast, the intact cells and cell walls retained much of their toxic properties even after prolonged heating. The response of the disrupted cells to heat treatment paralleled that of the cytoplasm for the first 10 min but leveled off with further heating. The intracytoplasmic extract was the most severely affected by heat treatment at 80 C (Fig. 3) suggesting the presence of heat-labile toxic

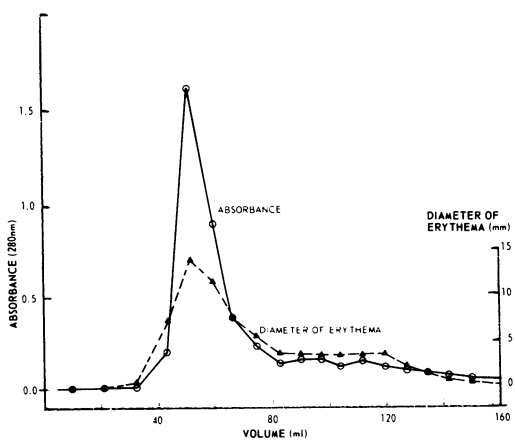


FIG. 2. *Sephadex G-200 gel filtration pattern of erthmal activity of F. necrophorum intracytoplasmic extract.*

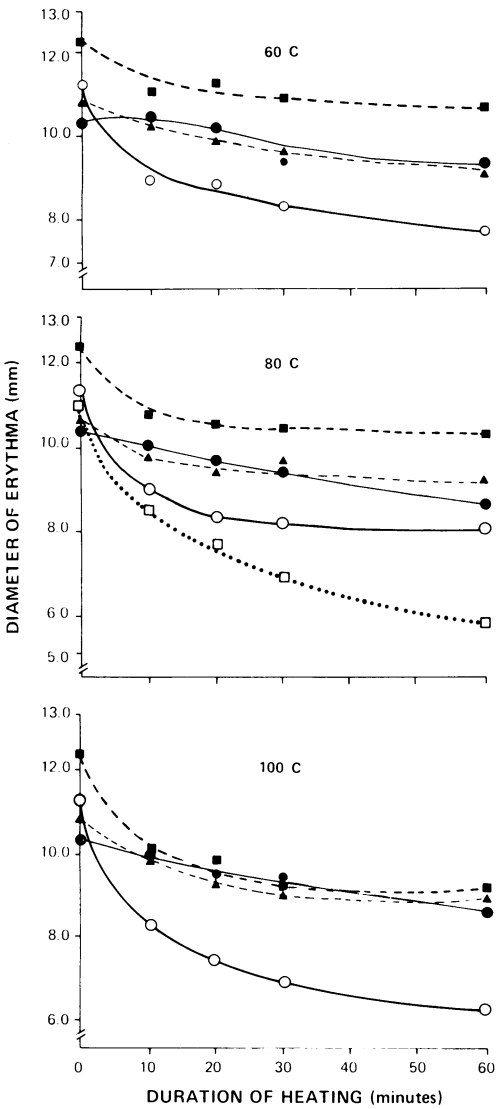


FIG. 3. *Effect of heat treatment on the erythemal activity of F. necrophorum cells and cell fractions. Symbols: ●, intact cells; ■, sonic extract; ▲, cell walls; ○, cytoplasm; □, intracytoplasmic extract.*

protein. The residual erythemal activity of the intracytoplasmic extract after prolonged heating might be due to relatively heat-stable protein component of the extract. The probability that the same could be due to traces of endotoxins was ruled out by the absence of positive gel formatiom in tubes of intracytoplasmic extract tested with the limulus assay for endotoxins (Tentative Technical Bull. 210. 1973. Sigma Chemical Co., St. Louis, Mo.). The resistance of the intact cells to heating might be related to the cell wall fraction as evidenced by the analogous trends of the erythemal activities of both preparations at the three temperatures and various heating times. Tissue culture assay of the heat-treated samples indicated a similar response but less sensitive than the intradermal test. For instance, no cytopathic effect was observed when the cytoplasm was heated at 100 C or at 80 C for more than 10 min. Although both assays gave reliable indications on the heat sensitivity of the different cellular fractions, the intradermal tests performed better than the tissue culture as a quantitative test.

**Precipitin reactions.** Rabbit antiserum against *F. necrophorum* intact cells formed at least two precipitation lines with its homologous antigen and with the sonic extract and cytoplasm (Fig. 4A) and one distinct band with intracytoplasmic extract. Serum from rabbits injected with the cell walls did not show any precipitating antibodies against its homologous antigen nor against the other bacterial antigens. The anti-cytoplasm serum, on the other hand, reacted quite strongly with the different antigen preparations (except cell walls) and showed two additional lines not revealed by the antiserum against intact cells (Fig. 4B). Final results from immunodiffusion plates of various

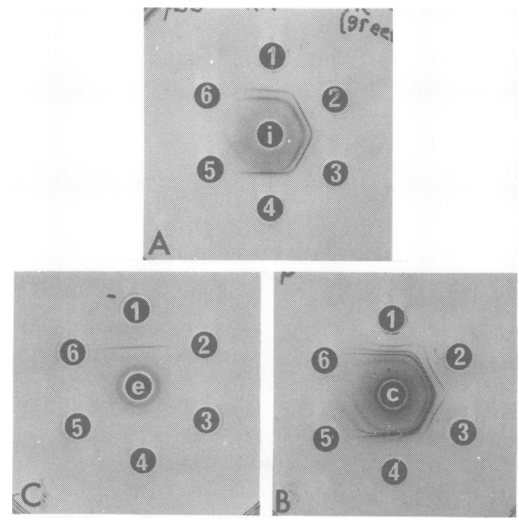


FIG. 4. *(A and B) Immunodiffusion assays of F. necrophorum cells and fractions. Center walls are hyperimmune sera against (i) intact cells and (c) cytoplasm. Peripheral wells are antigens (1) intact cells, (2) sonic extract, (3) cytoplasm, (4) intracytoplasmic extract, and (5) cell walls. Well (6) containing saline serves as control. (C) Effect of heating on the immunogenicity of intracytoplasmic extract. Center well is the anti-intracytoplasmic extract serum. Peripheral wells consist of the unheated intracytoplasmic extract (1) or heated at 60 C for (2) 10 min, (3) 20 min, (4) 30 min, (5) 60 min, and (6) saline.*

pH levels and incubated at different temperatures were similar. The heat sensitivity of the intracytoplasmic extract was further demonstrated by the loss of its characteristic antigenicity when exposed to 60 C for 10 min (Fig. 4C).

## DISCUSSION

Ultrasonic cell disruption used concurrently with differential centrifugation technique was effective in obtaining *F. necrophorum* cellular and subcellular toxic components. Although this fractionation method has been applied successfully in characterizing other bacterial toxins, it had not been previously exploited in studies of *F. necrophorum*. Admittedly, the need for adequate amounts of crude cells as starting materials for extensive fractionation and the apparent difficulty of growing the organism in bulk may have discouraged earlier workers from employing the above approach. However, these problems have been resolved by a simplified technique designed for the mass cultivation of the organism (9). Reports indicating that the toxins of *F. necrophorum* strains are mainly extracellular and that acetone-washed cells exhibit very low potency (6, 20) also may have deterred further attempts to search for cell-bound toxins. In fact, we have tried acetone treatment and found it too drastic as a means of obtaining an intracellular toxic fraction. By contrast, ultrasonic disruption not only retains the overt toxicity of the intact cells but, in some instances, enhances their effectiveness, probably as a consequence of the immediate contact between the undenatured cell-bound toxic components and the host tissues.

Certain salient features of the fractionation procedure related to obtaining relatively homogeneous preparations are noteworthy. Intact cells were washed twice prior to disruption, thereby minimizing contamination with constituents of the culture medium. Similarly, membrane filtration of the used culture medium freed the culture filtrate from cellular materials other than those derived from cells autolyzed during growth. The contribution of the latter, however, was not evident since the culture filtrate did not cause any significant toxicity. Electron microscopic examination of the cytoplasm did not detect any cellular materials resembling intact cell walls. The cleaned cell wall preparations were obtained without the use of potentially drastic treatments, viz., strong organic solvents and certain enzymes. The washing procedure employed in this study adequately freed the cell wall preparations of most of the nucleic acid-containing materials. Infrared spectrum of cell walls obtained from different batches exhibited uniformity and showed a principal band with an extinction at 1,660 $cm^{-1}$, corresponding to an amide-I band (3). The fractionation employed in this study, therefore, represents a straightforward and reliable first step to the eventual purification and biochemical characterization of *F. necrophorum* toxins.

Data from the various bioassays suggest that, under the growth conditions employed, the toxic factors of *F. necrophorum* are mainly cell bound. The variety of syptoms provoked by the different cellular and subcellular fractions of the pathogen is suggestive of the complexity of the toxic components. At least two cellular constituents are closely associated with these components: the cell walls and the cytoplasm. The cell wall-associated toxic component is relatively heat stable, and, aside from causing lethal and cytotoxic effects to mice and PKC, respectively, induces necrotic lesions on guinea pigs skin. Recently, we demonstrated the presence of serologically active lipopolysaccharide-protein preparations with strong toxic properties from *S. necrophorus* cell wall preparations (7). Cell walls of gram-negative bacteria have been known to possess a lipopolysaccharide-protein complex which is toxic and highly antigenic (3, 18).

The toxic component located in the cytoplasm is precipitated by ammonium sulfate, is strongly antigenic, is relatively heat labile, and has hemolytic property. It seems to be composed mainly of protein and, indeed, it purified and rendered more toxic could probably be classified as an intracytoplasmic toxin (18). Because of its association with the nondialyzable intracytoplasmic extract and its sedimentation and gel filtration properties, the intracellular toxic factor might be either a large-molecular-weight protein by itself or is strongly bound to high-molecular-weight, nontoxic proteins. Although the main protein toxic factor is heat labile, at least a small part of this complex protein may be relatively heat stable as shown by the skin test results. We suspect that this compound also may be less antigenic as no immunodiffusion line (Fig. 4C) appeared when the intracytoplasmic extract was heated at 60 C. To our knowledge, this report appears to be the first to show that *F. necrophorum* contains a protein toxic factor of intracellular origin.

Although there have been claims that animals cannot be protected against *F. necrophorum* infections by passive or active immunization (13,23), the strong immunogenic-

ity of the cytoplasm may have practical implications. Results of our 1972–73 filed trial (8) indicated that vaccination of beef animals with an alum-precipitated toxoid derived from the cytoplasm, at 15.5 mg of protein, reduced markedly the incidence of liver abscesses and scars to 10% compared with an average of 35% incidence in the uninoculated and adjuvant inoculated control groups. Furthermore, this toxoid reduced the number of abscesses per infected liver compared to the toxoided sonic extract and the control groups. These results, however, are preliminary and only suggestive at this time. In addition, we have unpublished data showing that sera from cattle vacinated with the cytoplasmic toxoid are inhibitory to the growth of *F. necrophorum*.

At best, the nature and mode of action of the *F. necrophorum* cell-bound toxins can only be speculated until such time as when the toxic components are purified and characterized. Nevertheless, the probable occurrence of two cell-bound toxins makes it conceivable that both may act complementarily in vivo, probably not unlike toxic mixtures (18). For instance, although individually the cell wall or the cytoplasm is toxic, its quantitative effect is never as severe as that obtained with the intact or disrupted cells. Thus, the overt toxicity is maximized when both fractions are present. Studies are currently underway to purify these toxic entities and to elucidate the mechanisms of the activities.

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