THE PATHOGENIC SYNERGY OF *FUSIFORMIS NECROPHORUS* AND *CORYNEBACTERIUM PYOGENES*

I. INFLUENCE OF THE LEUCOCIDAL EXOTOXIN OF *F. NECROPHORUS*

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Fusiformis necrophorus is often present in mixed infections but its pathogenic significance in relation to the other infecting organisms is not always apparent (Wilson and Miles, 1964). *F. necrophorus* is the predominant organism and probably the chief pathogen in ovine foot-abscess, although frequently *Escherichia coli* and *Corynebacterium pyogenes* are also present (Gregory, 1939).

Two forms of foot-abscess have recently been distinguished—lamellar suppuration, and infective bulbar necrosis (IBN) which is a mixed infection with *F. necrophorus* and *C. pyogenes*. In experimental mixed infections in sheep and guinea-pigs there is a synergy of *F. necrophorus* and *C. pyogenes*, with more rapid growth of each organism and greater inflammation and necrosis of the tissues than in pure infections with either organism alone. *F. necrophorus* appears to be the primary invasive and necrotizing agent with *C. pyogenes* participating less directly in the pathogenic process (Roberts, Graham, Egerton and Parsonson, 1968).

This is the first of 2 papers describing the separate contribution of each organism to the synergic relationship. It is shown that *F. necrophorus* facilitates the establishment and growth of *C. pyogenes* in the tissues through the leucocidal action of its exotoxin. The second paper contains an account of the way in which *C. pyogenes* stimulates invasion of the tissues by *F. necrophorus*.

MATERIALS AND METHODS

Organisms.—Two strains of *F. necrophorus* and 3 of *C. pyogenes* were isolated from the feet of sheep with ovine interdigital dermatitis (Parsonson, Egerton and Roberts, 1967), IBN, or lamellar suppuration. The strain of *Dermatophilus congolensis* was isolated from a lesion of ovine cutaneous actinomycosis.

Nutrient broth medium.—Oxoid " Lab Lemco " meat extract 0·5 per cent, Difco " Proteose Peptone " 1·0 per cent, Difco yeast extract 0·2 per cent, NaCl 0·5 per cent, and sufficient NaOH to give a pH of 7·6 before autoclaving.

Hoof broth medium.—Two per cent washed, powdered ovine hoof was added to the nutrient broth medium. The resulting suspension was autoclaved at 120° for 25 min. After cooling, 1 per cent Difco " Trypsin 1 : 250 " was added as a source of pancreatic growth factors and the medium was autoclaved at 110° for 15 min. After filtration to remove the particles of hoof, the pH was adjusted to 7·4. Shortly before inoculation, 0·1 per cent sodium thioglycollate was added and the medium was autoclaved at 105° for 10 min.

Horse red cell agar.—A suspension of washed horse red blood cells in physiological saline was added at a concentration of 5–7 per cent, to nutrient broth medium containing 1·6 per cent Difco " Bacto Agar ".

Broth cultures of F. necrophorus.—Volumes of hoof broth medium (5 ml.) were inoculated with 2 drops of a recent broth culture and incubated for 18 hr. at 37° in an atmosphere of 10 per cent CO_2 in H_2.

Sterile filtrates of F. necrophorus *broth cultures.*—Immediately after incubation the broth cultures were centrifuged at 2°, and the supernatant fluid was passed through sterile Millipore filters of 0·22 μ mean pore diam.

Broth cultures of C. pyogenes.—Volumes of hoof broth medium (5 ml.) were inoculated with *C. pyogenes* from cultures on blood agar and incubated for 17 hr. at 37° in an atmosphere of 10 per cent CO_2 in air.

Experimental injections.—Areas on the backs of animals, usually in groups of 4, were depilated by clipping. The materials were injected i.d. into the depilated areas in volumes of 0·1 ml. with 26 gauge needles.

Histological preparations.—Pieces of skin were fixed overnight in Bouin's solution. Paraffin sections were stained with Giemsa.

Heat-killed Staphylococcus aureus. *Staph. aureus* from cultures on blood agar were heated in physiological saline at 80° for 30 min., and then washed in 2 changes of saline.

Suspensions of zoospores of D. congolensis.—Broth cultures of *D. congolensis* were passed through coarse cellulose filter pads (" Ekwip " DS grade), which retained the mycelium and yielded virtually pure suspensions of zoospores.

Depletion of granulocytes with nitrogen mustard.—" Mustine Hydrochloride " (Boots), 2 mg./kg., was injected i.v. into rabbits lightly anaesthetized with nembutal. The rabbits were used 4 days later when their circulating granulocytes counts were less than 400 per c.mm.

Vaccination.—Broth cultures of *F. necrophorus* were killed by the addition of 0·5 per cent formalin. Rabbits were given 2 i.m. doses at weekly intervals of a killed culture emulsified in Freund's complete adjuvant, and then 2 i.v. doses at weekly intervals of fresh cultures to which formalin was added 1 hr. before injection.

Statistics.—The significance of differences was obtained by means of Student's t test.

RESULTS

The inflammatory reaction to F. necrophorus *and its influence on infection with* C. pyogenes *and other organisms*

The reaction of rabbits to cultures of F. necrophorus.—In rabbits the injection of broth cultures of *F. necrophorus* induced reddened swellings about 4 cm. in diam. at 5 hr. On incision the inoculated area was found to be surrounded by a thin layer of white material, and to contain numerous bubbles, suggesting that *F. necrophorus* produces gas in the tissues as it does in broth cultures. The layer of white material was seen in sections to consist of a dense accumulation of leococytes. Outside this layer leucocytes, mainly polymorphonuclears (PMN), were distributed fairly evenly through the tissues, but inside it there were no leucocytes. Numerous large spaces inside and outside the inoculated area had apparently resulted from the production of gas by the organism (Fig. 1). Growth of *F. necrophorus* was indicated by the presence of long, deeply staining filamentous forms towards the periphery of the inoculated area.

The leucocytes gathered around the injection site were damaged, with almost complete loss of structure and affinity for stain (Fig. 2). Apparently leucocytes entering the tissue in response to the injection were immobilized and killed when they came within close range of the inoculum, so that they accumulated at the periphery of the inoculated area. The absence of leucocytes inside the area suggested that the emigration of leucocytes was inhibited in vessels directly in contact with the inoculum. This was confirmed in a comparison of sections taken at 1, 3 and 5 hr., which showed that numerous PMN leucocytes had started to pass through the walls of these vessels, but had not completed the passage. At 24 hr. the vessels within the area were thrombosed and invaded by *F. necrophorus.*

The reaction of sheep and guinea-pigs to cultures of F. necrophorus.—The reaction of sheep and guinea-pigs was similar to that of the rabbit although there were differences of degree. The leucocytes of the guinea-pig were relatively resistant to the toxic effect of the culture so that, at 5 hr., they were more loosely accumulated at the periphery of the injection site, and showed less evidence of destruction than the leucocytes of the rabbit. In addition, there was very little growth of *F. necrophorus*, most of the injected organisms showing loss of structure and affinity for stain (Fig. 3). In sheep at 5 hr., the destruction of leucocytes and the growth of *F. necrophorus* were greater than in guinea-pigs but less than in rabbits.

A reaction observed only in guinea-pigs was necrosis of the epidermis above the injection site. PMN leucocytes were accumulated under the affected epidermis and some of them had infiltrated the necrotic tissue.

The reaction of rabbits and guinea-pigs to the cellular and fluid fractions of F. necrophorus *cultures.*—In rabbits at 5 hr. the reaction to *F. necrophorus* removed from broth cultures by centrifugation and resuspended in sterile hoof broth or 10 per cent hoof broth in buffered saline, was macroscopically and histologically the same as the reaction to the whole cultures. In addition, there was similar growth of the organism. The filtered supernatant fluid induced a less severe reaction, with erythematous swellings about 3 cm. in diam. at 5 hr. In this case there was a moderate, diffuse infiltration of PMN leucocytes which was slightly more dense than in sites where sterile hoof broth was injected; there was no evidence of a focal accumulation.

In guinea-pigs the resuspended organisms induced only a slight inflammatory swelling. In sections there was as little evidence of growth of the organism as in the sites of injection of whole cultures; the accumulation of leucocytes around the inoculum was just as intense, but there was no necrosis of the epidermis. The filtrates, by contrast, induced swellings of similar size and redness to those due to whole cultures, and the necrosis of epidermis was just as severe and extensive. The only focal leucocytosis was a slight infiltration of PMN leucocytes beneath and within the necrotic epidermis (Fig. 10).

It follows from these observations that the attraction of leucocytes to the site of injection of *F. necrophorus* cultures was due more to the organisms than to the fluid fraction. In the rabbit the toxic effect of organisms alone was not determined because *F. necrophorus* grew rapidly and probably produced soluble toxic substances in the dermis. However, organisms killed by heating at 60° for 30 min. and suspended at their initial concentration in fresh medium, induced only a very mild, diffuse inflammatory response; at 5 hr. there was merely a focal accumulation of leucocytes. In the guinea-pig, in which there was virtually no growth of the organism and therefore probably only slight production of toxic substances, the irritant and necrotizing effects were associated mainly with the fluid fraction. It seemed likely therefore that the irritant and cytotoxic actions of *F. necrophorus* cultures were due to an exotoxin.

The reaction of rabbits to heat-killed Staph. aureus *suspended in* F. necrophorus *culture filtrates.*—Heat-killed *Staph. aureus* was suspended in sterile hoof broth or the filtrate of a culture of *F. necrophorus*, at a concentration equivalent to Brown's tube 4, and injected into rabbits. In sections of tissue taken at 5 hr. there was an aggregation of PMN leucocytes at the site of injection of the suspension in the broth medium. Where the suspension in the filtrate was injected, however, there was an extensive area free of leucocytes but surrounded by a band of accumulated

PMN leucocytes. The accumulation was not as dense as that observed where living *F. necrophorus* was injected, nor were the leucocytes as severely damaged, but it was not expected that they would be without the continued production of toxin apparently associated with the rapid intradermal growth of *F. necrophorus*.

Since neither the sterile medium nor the filtrate alone induced an intense leucocytosis, it was clear that the attraction of PMN leucocytes to the injected suspensions was due almost entirely to the killed *Staph. aureus*, and that the immobilization of the leucocytes around the periphery was due to the leucocidal exotoxin of *F. necrophorus* in the filtrates. The killed bacteria at the centre of the injected area were thus protected against phagocytosis.

The intradermal fate of D. congolensis *zoospores suspended in* F. necrophorus *culture filtrates.—D. congolensis* infections are confined to the epidermis because of the organism's great susceptibility to the actions of granulocytes in the dermis (Roberts, 1965). *D. congolensis* was chosen, therefore, for an experiment to determine whether *F. necrophorus*, through the leucocidal action of its exotoxin, could facilitate infection with organisms susceptible to phagocytosis. Zoospores

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EXPLANATION OF PLATES

The photographs are all of skin sections stained with Giemsa.

FIG. 1.—Leucocytes arrested at the periphery of the site of intradermal injection of a culture of *F. necrophorus* in a rabbit at 5 hr. There are no leucocytes visible in the injected area (at right). The numerous large vacuoles probably contained gas produced by the organism. ×35.

FIG. 2.—At higher magnification long, deeply stained filaments of *F. necrophorus* can be seen to have grown near the band of arrested leucocytes at the edge of an area injected as in Fig. 1. Destruction of the leucocytes (at left) is indicated by the loss of structure and staining. ×305.

FIG. 3.—The periphery of the site of injection of a culture of *F. necrophorus* in a guinea-pig at 5 hr. Compared to those in the rabbit (Fig. 2) the injected organisms show poor structure and staining and apparently have not grown, and the arrested leucocytes are more scattered and show less evidence of damage. ×245.

FIG. 4.—The site of injection of a suspension of *D. congolensis* zoospores in hoof broth medium in a rabbit at 5 hr. The zoospores have been phagocytosed and have not formed hyphae. (The pallor of the leucocytes is the result of under-staining to show the zoospores more clearly.) ×440.

FIG. 5.—The site of injection of a suspension of *D. congolensis* zoospores in a filtrate of a culture of *F. necrophorus*, in a rabbit at 5 hr. In the absence of leucocytes the zoospores have germinated and formed hyphae. ×440.

FIG. 6.—A few poorly staining phagocytosed *C. pyogenes* within the accumulation of leucocytes in a sheep at 24 hr. at the periphery of an area injected with a broth culture of *C. pyogenes*. ×305.

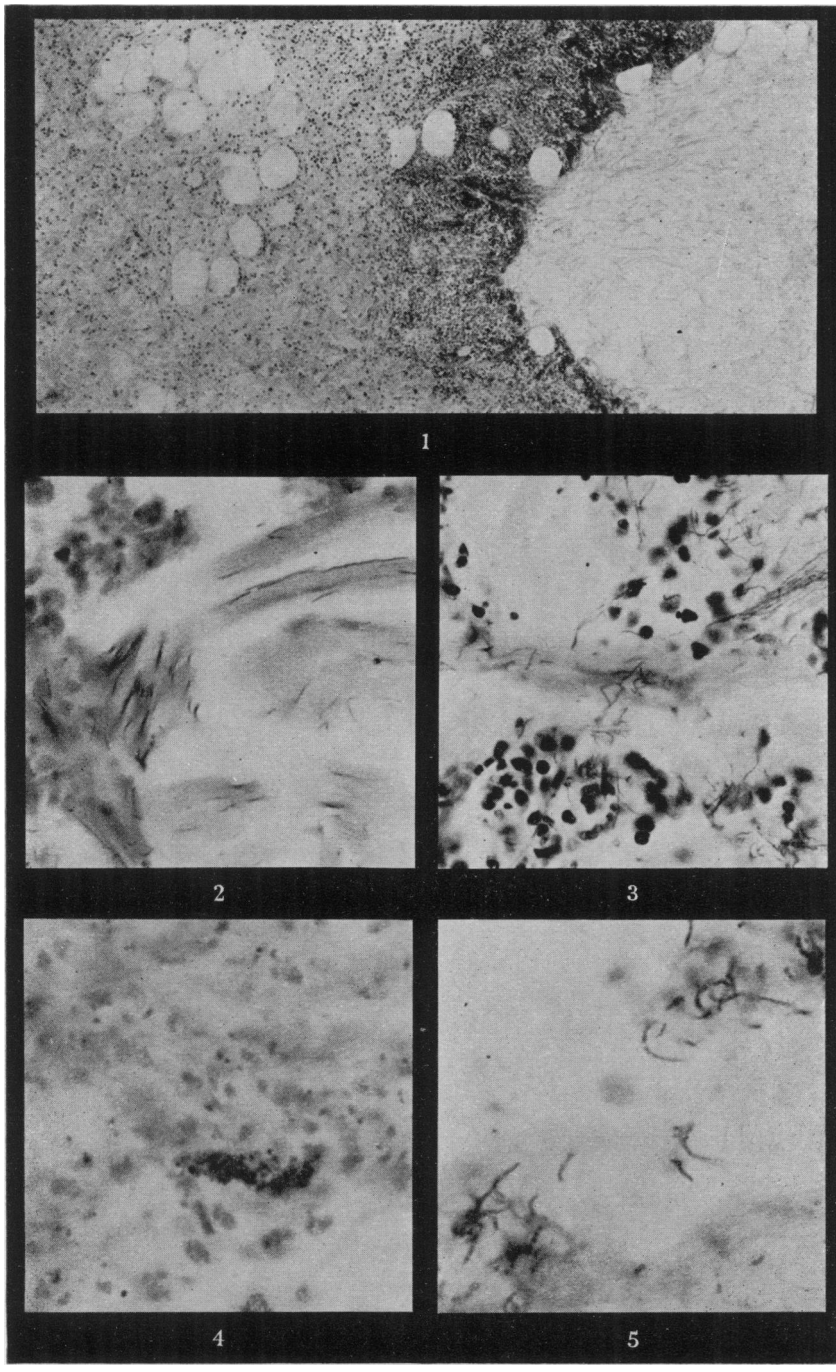
FIG. 7.—The site of injection of a mixed inoculum, in a sheep at 24 hr., showing three microcolonies of *C. pyogenes* and long filaments of *F. necrophorus* that have invaded the dense accumulation of leucocytes. The leucocytes have been almost completely destroyed and *C. pyogenes* has grown freely, compared to those shown in Fig. 6. ×305.

FIG. 8.—At lower magnification, numerous scattered microcolonies like those in Fig. 7 may be seen at 24 hr. after the injection of *C. pyogenes* in the dermis of a rabbit depleted of granulocytes with nitrogen mustard. ×120.

FIG. 9.—An area in the dermis of a rabbit injected with *F. necrophorus* suspended in homologous antiserum. At 5 hr. the antiserum has not prevented destruction of the accumulated leucocytes although the organisms have been inhibited from growing, and probably killed (see Fig. 2). ×305.

FIG. 10.—Necrosis of the epidermis above the site of intradermal injection of the filtrate of a culture of *F. necrophorus* in an unimmunized guinea-pig at 5 hr. ×120.

FIG. 11.—The site of intradermal injection of the filtrate of a culture of *F. necrophorus* in a guinea-pig given 5 ml. homologous antiserum intraperitoneally 18 hr. previously. At 5 hr. the epidermis above the site has been necrotized as completely as it was in the unimmunized controls (Fig. 10). ×120.



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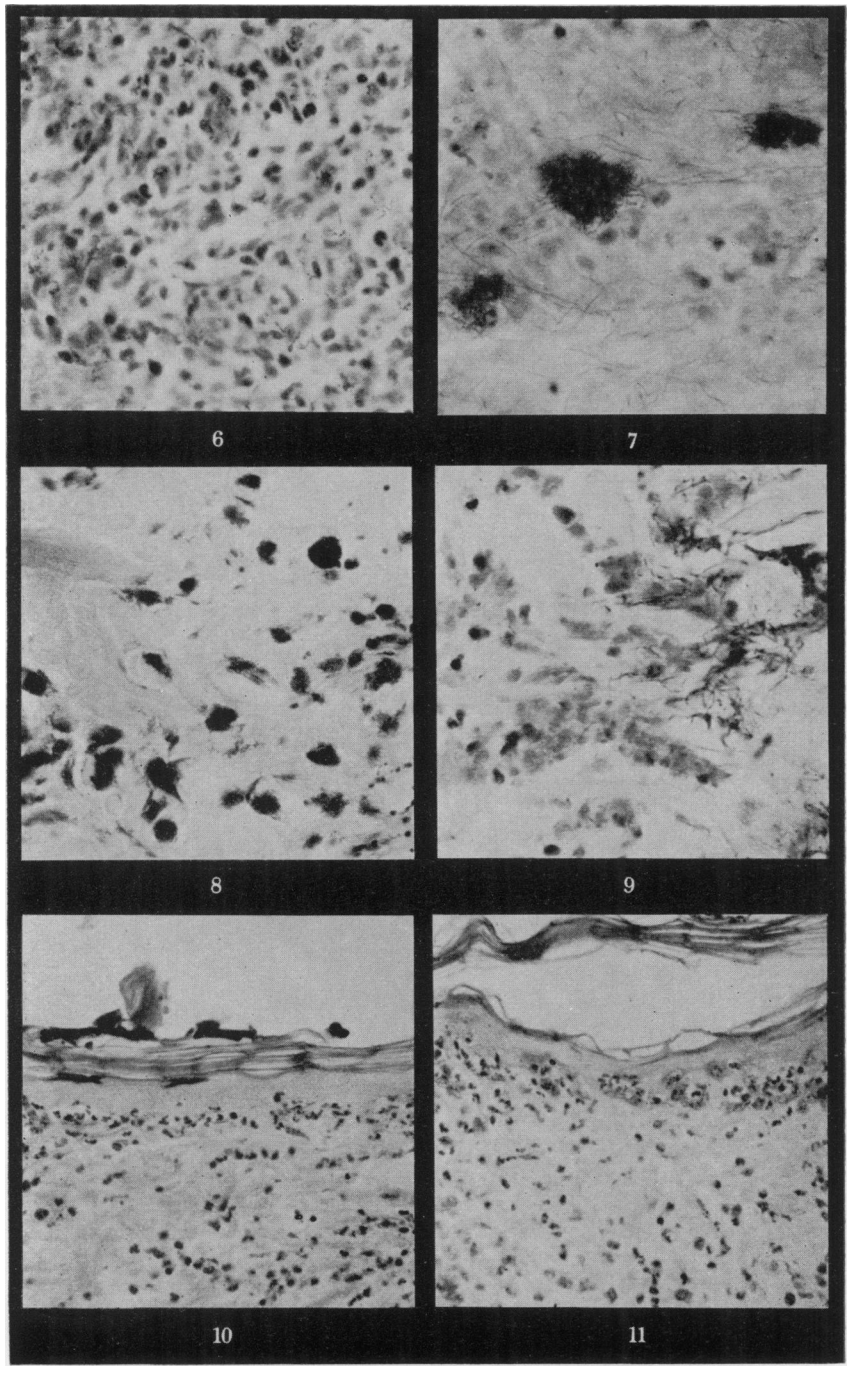

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of *D. congolensis* were suspended in either sterile hoof broth or the filtrate of a culture of *F. necrophorus*, at a concentration equivalent to Brown's tube 4. The suspensions were injected into rabbits and skin samples were taken at 5 hr.

The zoospores injected in the sterile medium were seen in sections to have been phagocytosed and prevented from growing (Fig. 4). The area where the zoospores in the filtrate were injected remained free of leucocytes. Within this area large numbers of zoospores had budded and produced fairly long hyphae (Fig. 5). Although there is no evidence that *F. necrophorus* and *D. congolensis* are ever found together in naturally infected tissues, this result supports the view that *F. necrophorus*, through the leococidal action of its exotoxin, could protect other organisms in a mixed infection.

The intradermal fate of C. pyogenes *suspended in* F. necrophorus *cultures and filtrates.*—Sections were taken at intervals after the injection of *C. pyogenes* broth cultures into rabbits, sheep and guinea-pigs. At 5 hr. in all 3 species the injection site was relatively free of leucocytes but the arrest and destruction of PMN leucocytes at the periphery were far less pronounced than in the experimental infections with *F. necrophorus*. Numerous *C. pyogenes* were visible within phagocytes in the inoculated area and at the periphery. At 24 hr., in each species, the infection was very limited in extent with a few apparently viable organisms free in the central area and the rest phagocytosed and undergoing destruction within PMN leucocytes (Fig. 6).

When *C. pyogenes* was injected into rabbits and guinea-pigs as a suspension in either a whole culture or a culture filtrate of *F. necrophorus*, the area free of leucocytes at 5 hr. was much more extensive and very few *C. pyogenes* were phagocytosed, compared to areas injected with the unmodified pure cultures of *C. pyogenes*. When *C. pyogenes* was injected intradermally into sheep as a suspension in a whole culture of *F. necrophorus*, sections of skin taken at 24 hr. showed a broad accumulation of leucocytes extending well beyond the edge of the injection site. The mass of leucocytes was infiltrated by filaments of *F. necrophorus* and the leucocytes were destroyed. Numerous microcolonies of *C. pyogenes* were clearly evident outside the injected area among the destroyed leucocytes, in contrast with the restriction of *C. pyogenes* at 24 hr. in the pure infections with this organism (Fig. 7). It was thus apparent that the destruction of leucocytes by the exotoxin of *F. necrophorus* could facilitate invasion of the tissues by *C. pyogenes*.

The effect of depletion of granulocytes on the proliferation of C. pyogenes *in the dermis of rabbits.*—Rabbits depleted of granulocytes with nitrogen mustard were injected with a broth culture of *C. pyogenes*. In sections of skin taken at 5 hr. there was no evidence of a PMN leucocytic response or of appreciable bacterial growth. At 24 hr. there were still no PMN leucocytes in the infected tissue although there was a moderate mononuclear infiltration at the periphery of the lesion. At this stage, however, numerous microcolonies of *C. pyogenes* could be seen extending well beyond the injected area (Fig. 8) in contrast with the restricted growth in undepleted controls. This confirmed that the restriction of pure infections with this organism is due to the presence of PMN leucocytes.

The effect of F. necrophorus *culture filtrates on the infectivity of* C. pyogenes *for rabbits.*—*C. pyogenes* was suspended in sterile hoof broth or culture filtrates of *F. necrophorus*, as either 10-fold or 2-fold serial dilutions starting at 10^8 per ml. The diluted suspensions were injected into rabbits. The presence of active infection was determined at each injection site at 2 days.

When suspended in the filtrates a concentration of 10^7–10^8 *C. pyogenes* per ml. was required to induce infection. In the sterile medium an average of twice as many organisms was required to induce infection in one experiment and 3 times as many in another. The infectivity of *C. pyogenes* was thus increased 2–3-fold in the presence of the leucocidal exotoxin of *F. necrophorus*.

The nature of the exotoxin of F. necrophorus

The association of the leucocidal and irritant actions of the exotoxin.—When cultures and culture filtrates of *F. necrophorus*, and suspensions of heat-killed *Staph. aureus* in the filtrates, were injected into rabbits depleted of granulocytes with nitrogen mustard, there was very little inflammatory response at 5 hr. to any of the injected preparations. This was in sharp contrast with the severe reactions to all the preparations in undepleted rabbits, and suggested that the irritant effect of the exotoxin is largely dependent on the presence of PMN leucocytes, and that it is mediated by the inflammatory products (Weissmann and Thomas, 1964; Thomas, 1964) of leucocytes destroyed by the exotoxin.

This was indicated most clearly in the response to injections of killed staphylococci suspended in *F. necrophorus* filtrates. On injection into normal rabbits, killed staphylococci in sterile hoof broth attracted large numbers of PMN leucocytes but induced only a slight macroscopic reaction, whereas the filtrates on injection attracted far fewer leucocytes but induced more severe swelling and erythema. The addition of killed staphylococci to the filtrates increased the diameter of the inflammatory swelling by an average of 6 ± 1 mm. in 1 experiment ($P < 0{\cdot}01$) and 7 ± 2 mm. in another, which would be equivalent to an increase in toxin concentration of more than 100-fold according to the regression of response to intradermally injected serial dilutions of filtrate in sterile broth. The increase in the inflammation probably resulted from the destruction by the exotoxin of the large numbers of PMN leucocytes attracted to the dead bacteria. This was supported by the finding that in rabbits depleted of granulocytes the addition of killed staphylococci caused no significant increase in the response to the filtrates (average increase 1 ± 1 mm.).

The inflammatory response to the filtrates was therefore considered a valid measure of their leucocidal activity and hence their content of exotoxin.

The dialysability and heat-stability of the exotoxin.—The irritant activity of *F. necrophorus* filtrates was determined after dialysis in Visking tubing for 2 days against 4 changes of sterile hoof broth, or after heating for 30 min. at 56° or 100°. The intradermal inflammatory response of rabbits to the filtrates was not diminished after dialysis. Moreover, the slight response to the sterile medium was not increased after it had been dialysed for 2 days against 4 volumes of filtrate. Therefore, the exotoxin is an undialysable macromolecule.

There was no apparent loss of activity at 56° but after heating at 100° the average diameter of the inflammatory swellings was decreased by $2{\cdot}5 \pm 1{\cdot}25$ mm. in one experiment, and $2{\cdot}1 \pm 0{\cdot}4$ mm. in another ($P < 0{\cdot}02$). A difference of 2·1–2·5 mm. is approximately equivalent to a 10-fold dilution or to a 90 per cent loss of activity. On the other hand, the reaction to filtrates heated at 100° exceeded the reaction to the sterile medium by $3{\cdot}8 \pm 1{\cdot}1$ mm. ($P < 0{\cdot}02$), and $5{\cdot}8 \pm 0{\cdot}4$ mm. ($P < 0{\cdot}001$), confirming that the inactivation was incomplete.

Association of exotoxic and haemolytic activity.—Fievez (1963) found that the haemolytic and pathogenic powers of different isolates of *F. necrophorus* were

strongly associated, and suggested that the haemolysin and exotoxin were identical. The haemolytic activity of heated and unheated filtrates was tested, therefore, by mixing with equal volumes of washed horse red blood cells in physiological saline and incubating at 37° for 2 hr. Unheated filtrates lysed the suspensions in 5–10 min. Filtrates heated at 56° for 30 min. induced no evidence of haemolysis in 2 hr., however, indicating that the exotoxin and haemolysin differ considerably in heat-stability.

The ability of strongly anti-haemolytic antibody to inhibit the toxic actions of the immunizing strain was also tested. Serum from rabbits vaccinated with formalinized *F. necrophorus* inhibited haemolysis completely when added at a concentration of 20 per cent to the mixtures of culture filtrate and washed red cells, and almost completely in cultures of *F. necrophorus* when 5 per cent of the antiserum was added to horse red cell agar; normal rabbit serum was not inhibitory at these concentrations. On the other hand, the inflammatory response to the injection of a 10-fold series of dilutions of a filtrate in sterile broth, was the same at each dilution in the vaccinated rabbits as in unvaccinated controls. In addition, in unvaccinated rabbits, the destruction of leucocytes was found histologically to be just as severe at sites injected with *F. necrophorus* suspended in the antiserum as in normal serum, although the organisms were destroyed by the antiserum as shown by their lack of growth and their loss of structure and affinity for stain (Fig. 9). Moreover, in 500 g. guinea-pigs given 5 ml. rabbit antiserum intraperitoneally 18 hr. earlier, the necrosis of epidermis at 5 hr. over the sites of intradermal injection of cultures or filtrates of *F. necrophorus*, was just as severe and extensive as in unimmunized controls (Fig. 10 and 11).

Therefore the exotoxin is not identical with the haemolysin and not neutralizable by the antibody of rabbits vaccinated with formalinized cultures.

The effect of F. necrophorus *on the growth of* C. pyogenes in vitro.—On nutrient agar, and in broth media under special conditions, *C. pyogenes* stimulates the growth of *F. necrophorus*, an effect to be discussed in more detail in the second paper. On the other hand, in the nutrient or hoof broth media, either in liquid form or solidified with agar, there was no evidence that *F. necrophorus* influenced in any way the growth of *C. pyogenes*.

DISCUSSION

An absence of leucocytic emigration in tissues invaded by *F. necrophorus* has been described in natural infections of cattle and sheep (Flint and Jensen, 1951; Roberts *et al.*, 1968). In the experimental intradermal infections with *F. necrophorus* described above the emigration of leucocytes was completely inhibited within the area where the cultures were injected; extravascular leucocytes outside the injected area were arrested and destroyed at the edge of it. The reproduction of these effects in rabbits, with sterile *F. necrophorus* filtrates containing heat-killed staphylococci to attract leucocytes, suggests that the factor responsible for the arrest and destruction of leucocytes is a toxin free in the culture medium. Effective concentrations were present in solution at 18 hr., which is probably too early for autolytic changes of a degree that would release endotoxins. Moreover, the cellular fraction of the cultures had very little leucocidal activity at 18 hr. The active substance is therefore considered to be an exotoxin.

The inability of cultures and filtrates of *F. necrophorus* to induce severe

inflammation in the skin of rabbits depleted of granulocytes, suggests that the irritant activity of the organism and its products in undepleted rabbits is largely due to inflammatory factors released during the destruction of PMN leucocytes (Weissmann and Thomas, 1964; Thomas, 1964). This is consistent with the synergy observed with suspensions of killed staphylococci in filtrates of *F. necrophorus*; although killed staphylococci in the sterile medium were less irritant than the filtrates, the dead organisms increased the irritant activity of the filtrates more than 100-fold. This synergic effect was probably due to the destructive action of the exotoxin on the numerous PMN leucocytes attracted to the site by the dead organisms, especially since it was abolished in rabbits depleted of granulocytes. The irritant activity of the filtrates was accordingly used as a measure of their exotoxin content.

Beveridge (1934) referred to intradermally irritant and intravenously lethal actions of an exotoxin of *F. necrophorus*, but did not investigate the nature of the toxin or the mediation of its effect on tissues. It has been suggested that the exotoxin is identical with the haemolysin (Fievez, 1963) but apparently they are different; the haemolysin was inhibited by antiserum, and inactivated by heating at 56°. The exotoxin, on the other hand, was not affected by either agent; about 10 per cent of the toxicity remained after 30 min. at 100°. The exotoxin is undialysable and is therefore macromolecular, but further study is needed to elucidate its chemical nature.

In both natural and artificial infections, *F. necrophorus* is apparently protected from phagocytosis as a result of the arrest and destruction of leucocytes by exotoxin diffusing from the invading filaments. It was to be expected, therefore, that in mixed infections *F. necrophorus* would protect other organisms from phagocytosis through the same mechanism. An effect of this kind was observed with suspensions of killed staphylococci in *F. necrophorus* filtrates; phagocytosis of the dead organisms was greatly decreased as a result of the arrest of cellular infiltration by the exotoxin.

The protective effect of the exotoxin was most clearly evident, however, with zoospores of *D. congolensis*. This organism is extremely susceptible to the phagocytic and other actions of granulocytes, so that infection is usually restricted to the epidermis. In rabbits depleted of granulocytes, on the other hand, it freely invades the dermis (Roberts, 1965). Whereas zoospores alone were phagocytosed and prevented from growing, those suspended in the filtrates of *F. necrophorus* cultures were able to germinate and form typical hyphae.

The poor *in vivo* growth of *C. pyogenes* is apparently due entirely to destruction of the organism by phagocytosis. However, though *F. necrophorus* filtrates enhanced infection with *C. pyogenes*, the effect was less pronounced than with *D. congolensis* zoospores because *C. pyogenes* itself has a slight ability to arrest and destroy leucocytes and to grow in the dermis; the infectivity of *C. pyogenes*, measured in terms of infecting dose, was increased only 2–3-fold by *F. necrophorus* filtrates. In the mixed infections, on the other hand, the continued destruction of leucocytes by freely proliferating *F. necrophorus* was associated with increased and sustained growth of *C. pyogenes* and invasion by this organism of the tissues beyond the injection site. The fact that an equivalent proliferation resulted when pure *C. pyogenes* was injected into rabbits depleted of granulocytes, confirmed that the effect of *F. necrophorus* on the growth of *C. pyogenes* was dependent on the destruction of PMN leucocytes by the exotoxin.

Since there was no *in vitro* evidence that *F. necrophorus* supplied a growth factor for *C. pyogenes*, the increased proliferation of *C. pyogenes* in the sites of mixed infection may therefore be attributed to inactivation of the phagocytic process by the exotoxin of *F. necrophorus*. Under natural conditions, the leucodial action of the exotoxin may also facilitate infection with other organisms susceptible to phagocytosis.

SUMMARY

In experimental infections with *Fusiformis necrophorus* in the dermis of rabbits, sheep and guinea-pigs, leucocytes were inhibited from leaving the blood vessels within the inoculated area, and were arrested and destroyed at the periphery on migration from outside the area. These effects were found to be caused by a leucocidal exotoxin released from the organism during growth.

The intradermal inflammatory action of the exotoxin was shown to be dependent on the presence of granulocytes in the circulation, and was increased more than 100-fold by the presence of dead bacteria that attracted leucocytes to the injection site. Apparently the inflammatory action of the exotoxin is due mainly to the release of irritant substances from leucocytes damaged by the toxin.

The exotoxin content of *F. necrophorus* culture filtrates was measured in terms of intradermal irritant activity. The exotoxin was found to be an undialyzable macromolecule slowly inactivated at 100°. It was not inactivated by heating at 56° or by strongly antihaemolytic antiserum, and is thus distinct from the haemolysin.

When other bacteria were injected as suspensions in *F. necrophorus* filtrates, they were protected from phagocytosis by the leucocidal action of the exotoxin and their growth in the tissues was thereby facilitated. In mixed infections with *F. necrophorus* and *Corynebacterium pyogenes* the increased growth and invasiveness of *C. pyogenes* was likewise attributable to prevention of phagocytosis by the exotoxin of *F. necrophorus*.

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