INFECTION AND IMMUNITY, June 1974, p. 1114-1122

Vol. 9, No. 6
Printed in U.S.A.

Cytotoxic Activity of *Mycobacterium ulcerans*

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Received for publication 26 December 1973

Although *Mycobacterium ulcerans* elicits extensive necrosis of human skin and subcutaneous tissue, the specific cause of the necrosis has never been elucidated. In an attempt to define a toxic substance, 18 strains of *M. ulcerans* were inoculated into mice, and the progress of each infection was observed and compared with infections of other mycobacteria, including *M. cheloni*, *M. marinum*, and *M. bovis*. Culture filtrates and viable organisms of each of these mycobacteria were inoculated onto tissue culture cells. Inoculation of mouse footpads with *M. ulcerans* resulted in progressive infections, leading to ulceration and eventual death. Strains of *M. cheloni*, *M. marinum*, and *M. bovis* did not produce progressive infections in the mice. Culture filtrates of *M. ulcerans* produced severe cytopathogenic effects on tissue cells, but washed, viable organisms of the same strains had no cytopathogenic effect. Culture filtrates and viable organisms of *M. cheloni*, *M. marinum*, and *M. bovis* did not produce a cytopathogenic effect on the tissue cells. Viable organisms of selected strains of *M. ulcerans* and the culture filtrates of the same strains were inoculated into guinea pig skin. The culture filtrates and the viable organisms both caused focal necrosis and focal inflammation, changes that resemble those in the naturally occurring infection in man. Preliminary purification of the culture filtrate by ultrafiltration indicates that the toxic fraction has a molecular weight of approximately 100,000, and temperature studies indicate that the toxic fraction is heat labile.

Mycobacterium ulcerans infects man in endemic proportions in some areas of Australia (9), Uganda (2), Zaire (7), Nigeria (6), New Guinea (12), and Malaysia (11). Sporadic cases have also been seen in Mexico (8) and the Congo (Brazzaville) (10). One patient who acquired her infection in Bolivia has been studied (Armed Forces Institute of Pathology [AFIP] accession no. 1383082; contributed by Wendell H. Hall), and one patient with a typical infection has been seen in Ghana (1). The disease is characterized clinically by chronic ulceration of the skin, with undermined edges. Microscope study reveals extensive necrosis of the dermis and subcutaneous adipose tissue, and large clumps of acid-fast bacilli are present in the necrotic tissue. Characteristically, the necrotic tissue extends beyond the organisms, and in one lesion the acid-fast bacilli were confined to a relatively small, central zone in a large area of necrosis (3). Because of this, Connor and Lunn (3, 4) suggested that the organism produces a diffusible toxin that devitalizes the tissues. To test this hypothesis, strains of *M. ulcerans* recently isolated from infected Zairians were collected, assayed for toxic substances, and compared with other mycobacteria as herein described.

MATERIALS AND METHODS

Cultures: isolation of organisms from Zairians. Strains of *M. ulcerans* were recovered from the ulcer margin of patients in Zaire. Swabs were rubbed on the necrotic exudates of the ulcers and then decontaminated in 5 ml of 5% oxalic acid at room temperature for 15 min. The swabs were withdrawn and the contents were centrifuged. The supernatant was decanted, and 1.0 ml of sterile saline and a drop of phenol red indicator were added to the sediment. This mixture was neutralized with 4% NaOH and, after 5 min, inoculated into six slants of Lowenstein-Jensen (L-J) medium (Difco). Two tubes were incubated at 37 C, two were incubated at 32 C, and two were incubated at room temperature (25 to 32 C).

Strains of *M. ulcerans* were also isolated from

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biopsy specimens. Epidermis and superficial dermis were cut away, and the deeper dermis and necrotic subcutaneous tissue were diced. About 1 g of this was ground in a Ten Broeck grinder with 3 to 5 ml of saline, or in Griffith tubes with sterile sand and saline. The slurry was centrifuged at slow speed to remove coarse particles, the supernatant was decanted, and twice its volume of 5% oxalic acid was added and left for 15 min. After centrifugation the supernatant was decanted, the sediment was resuspended, and the mixture was neutralized with 4.0% NaOH, inoculated onto six slants of L-J medium, and incubated as above.

Fractions tested for toxicity. The mycobacteria grown on L-J medium were subcultured in 5 ml of Dubos broth (Difco) containing 0.5% bovine albumin (grade B, fraction V; Pentes, Kankakee, Ill.). Incubated at 32 C and agitated several times a week, the cultures matured in 6 weeks at about 10^8 organisms per ml (Petroff-Hauser chamber). A 1-ml volume was inoculated into each of several 1-liter bottles containing 100 ml of Dubos medium. When these in turn became mature, they were pooled and centrifuged (1,500 × *g*) for 30 min at 5 C.

The supernatant was filtered (HA; 0.45 μm; Millipore Corp., Bedford, Mass.), yielding a culture filtrate; the remainder was discarded. Fractions of the culture filtrate were lyophilized; others were heated for 1 h at 60, 80, or 100 C; unheated samples were stored for 1 month at both 4 and −20 C.

The sedimented organisms (centrifugate) were suspended in 30 ml of water, chilled in ice, sonically treated for 1 h at 195 W (model S-74; Branson Instrument Co., Danbury, Conn.), and centrifuged (1,500 × *g*) for 30 min. Fractions of the sonically treated supernatant were heated at 60 C for 1 h; other fractions were filtered, as was the supernatant, yielding a "sonically treated filtrate."

Purification. Preliminary separation of the toxic substance from inactive substances was undertaken by dialysis (dialyzer tubing; A. H. Thomas Co., Philadelphia, Pa.) of 50 ml of the culture filtrate against 1,000 ml of water, three times at 4 C. The nondialyzable factor and part of the first dialysate were lyophilized, reconstituted appropriately, and tested for toxicity. Unheated culture filtrate was fractionated by pressure filtration with nitrogen through graded membrane filters (PM30 through XM300; Amicon Instruments, Lexington, Mass.) and also tested for toxicity.

Cell cultures. Suspensions of cloned L-929 cells (cloned in 1963 by Ilse Tribby from L-929 mouse fibroblasts and subsequently maintained on medium 199 with 10% fetal calf serum) were maintained in logarithmic growth in cell culture medium 199 (Hanks base) with 10% fetal bovine serum (Microbiological Associates, Bethesda, Md.) and antibiotics (penicillin, 100 U/ml; and streptomycin, 100 μg/ml). Semiconfluent monolayers were prepared by adding 5.0×10^5 cells to 25 cm^2 Falcon tissue culture flasks (Bioquest, Cockeysville, Md.) with 4 ml of growth medium. After overnight incubation, the growth medium was changed and 0.05 ml of test substance was added. Three flasks were used to test samples of each strain and sonically treated supernatants of some strains. In addition, the samples of culture filtrates and sonically treated supernatants and filtrates, heated and stored as already described, were tested for cytotoxic effects. Also, broth cultures of organisms were washed three times with Dubos medium, suspended in the original volume of fresh Dubos medium, and tested. Controls included flasks of L-929 cells with no inocula and flasks of L-929 cells inoculated with 0.05 ml of Dubos medium. Cell cultures were incubated at 37 C and examined daily for 72 h, and in some experiments they were incubated at 32 C and examined daily for 48 h. After examination by phase microscopy, the representative flasks were washed three times with 0.15 M phosphate-buffered saline (pH 7.2) and stained by the Giemsa technique.

The L-929 cells inoculated with the different test substances and incubated at 37 C showed degrees of cytopathic effect (CPE) classified from 0 (indicating no CPE) to 4+ (indicating over 80% of cells rounded and over 60% of cells detached).

Mice. One footpad of each mouse received an inoculum of 0.05 ml, and the time until ulceration and death was noted.

(i) Five young adult inbred white mice were inoculated with a mature culture of Dubos broth (10^8 organisms per ml) of each strain of *M. ulcerans* tested.

(ii) Logarithmic dilutions of a mature broth culture of one strain (70-22A MP) of *M. ulcerans* were inoculated into each of five young adult CBA/J mice.

All of the infected feet of the above mice were examined three times a week to determine the time of onset of ulceration. The infections were usually allowed to run their course, and the time of death was noted.

(iii) Ten inbred white mice were inoculated with a mature Dubos broth culture of *M. ulcerans*. Two sets of 10 mice were used as controls. One set received no inoculum; the other was inoculated in one footpad each with Dubos medium. Inoculated feet and control feet were measured by vernier calipers twice each week. The measurements were made vertically through the middle of the tarsus.

Guinea pigs. Each of eight guinea pigs weighing 250 to 350 g was inoculated with 0.1 ml of Dubos Tween 80 medium and 0.1 ml of old tuberculin (Jensen-Salsbery Laboratories, Kansas City, Mo.) diluted 1:100 at duplicate test sites as follows. (i) Three animals were inoculated with 0.1 ml of a mature Dubos broth culture of *M. ulcerans* containing 6.0×10^7 organisms of strain 70-22B per ml. (ii) Five animals were inoculated with 0.1 ml of culture filtrate and 0.1 ml of sonically treated supernatant of the same strain.

Histopathological observations. Guinea pigs inoculated intradermally with pasteurized, sonically treated supernatant and culture filtrate were killed at intervals of 2, 8, and 30 days after inoculation. Guinea pigs inoculated intradermally with viable *M. ulcerans* organisms were killed at intervals of 1, 2, and 5 days after inoculation. Specimens of skin from the sites of inoculation were fixed in 10% neutral, buffered formalin, processed, cut, and stained by routine techniques.

RESULTS

CPE of toxic fractions. Table 1 lists the mycobacteria studied and further identifies them by contributor's number, by AFIP accession number, and by the geographic area where the patient probably became infected. The final column indicates the CPE produced on L-929 cells by filtered toxic broth supernatants of each strain.

Table 2 lists the degree of CPE in samples from strain 70-22A of *M. ulcerans* organisms and also the CPE of culture filtrates and sonically treated supernatants heated or stored at various temperatures for various periods.

Maximal CPE developed in 48 h; further incubation up to 72 h had no additional effect on cells. Incubation at 32 C also yielded maximal CPE at 48 h. The L-929 cells in the control flasks with 0.05 ml of Dubos medium added and without inocula showed no CPE.

Relative virulence of M. ulcerans. Table 1, in addition to listing cytotoxicity, lists time in which ulcers developed. All mice died with continuing infections and, in 21 of 147 mice inoculated, the infected foot sloughed off. Extension of the infection to uninoculated feet was not observed, but in one animal the tail became ulcerated.

Figure 1 relates the number of organisms

TABLE 1. *Identification, source, interval until ulceration, and toxic effects of mycobacteria studied*

Strains of mycobacterium tested and contributor's no.	AFIP patient accession	Country of origin	Time (weeks) from inoculation to ulceration of mouse foot pad[a]	CPE produced by culture filtrate on L-929 cells
M. ulcerans				
70-22A	1352735	Zaire	5	4+
70-22A MP[b]	1352735	Zaire	4	4+
70-22B[c]	1352735	Zaire	18	4+
70-38	1356545	Zaire	19	0
70-40	1357478	Zaire	22	2+
70-59	1379274	Zaire	No ulceration	4+
70-60[c]	1379274	Zaire	4	Not tested
70-60 MP[b]	1379274	Zaire	12	2+
70-64	1392852	Zaire	5	4+
71-10	None	Zaire	No ulceration	4+
71-12	None	Zaire	No ulceration	4+
71-75	None	Zaire	4	4+
71-202	1393629	Zaire	3	4+
71-205	None	Zaire	3	4+
71-216	1403253	Zaire	3	4+
71-234	1404750	Zaire	4	4+
RF[d]	1228472	Nigeria	No ulceration	0
455[e]	1379392	Nigeria	5	4+
M. cheloni				
70-27	None	Zaire	Not tested	0
70-32	None	Zaire	Not tested	0
M. marinum				
E410[d]	None	U.S.A.	No ulceration	2+
Mycobacteria sp.[c]	1368895	U.S.A.	No ulceration	0
M. bovis (BCG)[f]	None		No ulceration	0

[a] All footpads were inoculated with 0.05 ml of a mature broth culture.

[b] This strain was re-isolated after one passage in the footpad of a mouse.

[c] Strains 70-22B and 70-60 of *M. ulcerans* and the *Mycobacteria* sp. (accession no. 1368895) from Bay Pines, Fla., were isolated at 37 C. All other organisms were isolated at 32 C.

[d] Contributed by Norman E. Morrison of the Leonard Wood Memorial Hospital, Leonard Wood Memorial Research Laboratory, Johns Hopkins School of Hygiene, Baltimore, Md.

[e] Isolated in 1971 from a Nigerian living in New York City; contributed by Farrington Daniels and Sonia Lindo of Cornell University Medical Center, Ithaca.

[f] Obtained from the National Institutes of Health in 1968 and maintained on Lowenstein-Jensen medium since then.

TABLE 2. *Effect of physical factors on the cytotoxicity of various substances from strain 70-22A (M. ulcerans)*

Substance	Expt	Treatment resulting in CPEs of:		
		4+	2+	0
Culture filtrate	1	Freshly harvested	Stored at −20 C for 1 month	Stored at 4 C for 1 month
	2	Heated to 60 C for 1 h	Heated at 80 C for 1 h	Heated at 100 C for 1 h
	3	Lyophilized for 1 week		
	4	Dialyzed		
Sonically treated supernatant	1	Freshly prepared		
	2	Heated at 60 C for 1 h		
Sonically treated filtrate		Freshly prepared		
Viable organisms				Washed 3 times

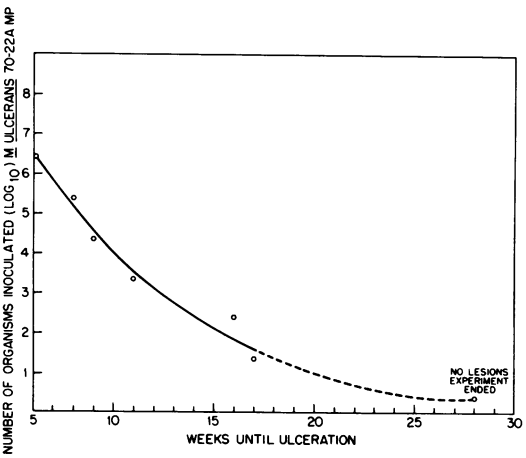

FIG. 1. *Number of organisms related to incubation period. Graph shows time from inoculation to ulceration in mouse footpads inoculated with logarithmic dilutions of M. ulcerans in broth culture.*

inoculated (strain 70-22A MP) to the time ulceration began in the footpad of the mouse.

Figure 2 illustrates the progress of *M. ulcerans* infection in white mice inoculated with 10^7 organisms. Nine of the ten mice inoculated died between 7 and 13 weeks later. The other mouse survived even after gangrene developed, and the foot sloughed off at 12 weeks; the animal was killed at 18 weeks. The thickness of the infected feet ranged from 2.5 mm at time of inoculation to 6.7 mm in animals surviving 11 weeks after inoculation. The thickness of the feet of control animals and the noninoculated hind foot of infected animals increased from 2.5 to 2.7 mm 11 weeks after inoculation. None of the animals in the control groups developed ulcers or died.

Ultrafiltration. A toxic effect was not evident in the filtrate from the XM50 membrane or in the material retained on a PM30 filter. A reduced toxic effect (2+ CPE) was evident in the residue on both the XM100 and the XM300 filter. The residue on both the XM100 and the XM50 filters produced 4+ CPE in 48 h on L-929 cells.

CPE on L-929 cells. Tissue cells exposed to sonically treated filtrate and supernatant and culture filtrate had a marked CPE. At 24 to 48 h after exposure to each of these preparations, the cells had decreased in number and size. In addition, their nuclei were pyknotic and displaced toward the margin of the cell or partially through the cell wall. Some were without nuclei. (Compare the control cells in Fig. 3 with the exposed cells in Fig. 4.)

Gross lesions. Guinea pigs inoculated intradermally with viable organisms of *M. ulcerans*, culture filtrate, or sonically treated supernatant developed focal lesions at the site of inoculation. Those receiving viable organisms developed focal erythema between days 1 and 4. These foci increased to between 5 and 10 mm in diameter during the first week. Between days 5 and 18, scabs formed over the center of the erythematous areas. These were 2 to 3 mm in diameter and became detached between 20 and 30 days, leaving a healed, re-epithelialized surface. Those receiving sonically treated supernatant developed focal erythemas between days 1 and 2 that were 8 to 10 mm in diameter. These foci decreased (5 to 8 mm) during the first week.

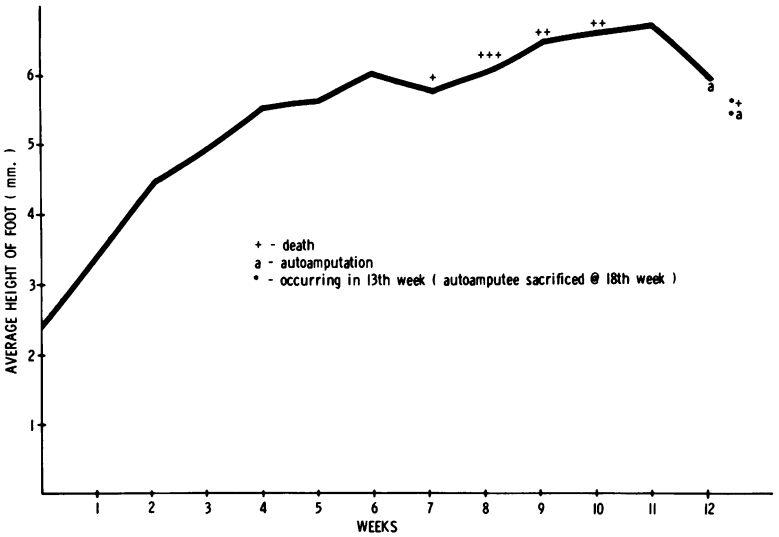

FIG. 2. *Death rate of mice and progress of swelling of feet.*

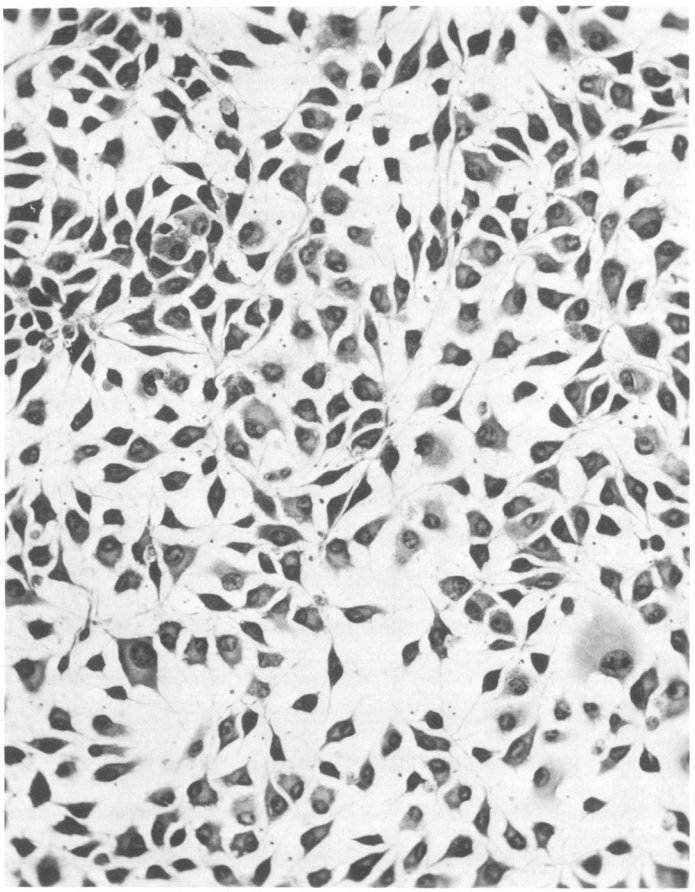

FIG. 3. *Monolayer of L-929 cells showing no CPE. These were incubated at 37 C for 48 h. Giemsa, ×195, AFIP negative 72-6555.*

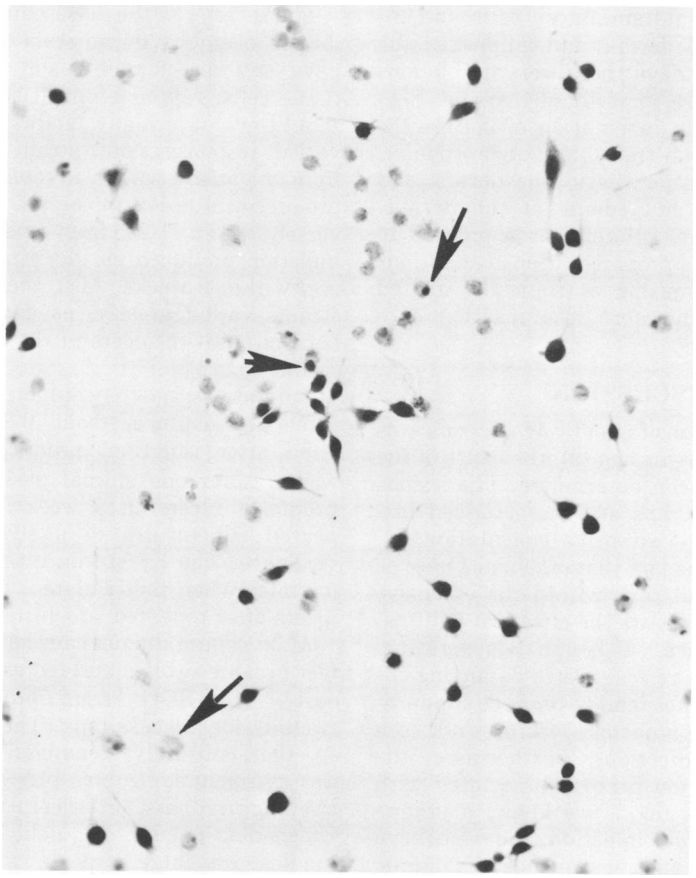

FIG. 4. *Monolayer of L-929 cells with culture filtrate added ana incubated at 37 C for 48 h. The effect of exposure to the toxic substance is clearly shown. The CPE includes a reduction in number and size of attached cells. The nuclei are shrunken and pyknotic. The nuclei of some degenerating cells (arrows) are missing or adherent to the cell margin. Apparently some nuclei have been extruded through the cell wall. Giemsa, ×195, AFIP negative 72-6558.*

Between days 6 and 9 small scabs (2 to 4 mm) formed on the surface and became detached between 10 and 20 days, leaving a healed, re-epithelialized surface.

Guinea pigs receiving culture filtrate developed focal erythemas between days 1 and 2, that were 7 to 10 mm in diameter. These foci disappeared during the first week.

At no time did the lesions weep, ulcerate, or slough necrotic tissue. None of the animals died. None of the guinea pigs reacted to tuberculin or Dubos medium.

Microscopic findings. Sections prepared from specimens taken 24 h after intradermal inoculations with viable organisms of *M. ulcerans* revealed edema of the dermis at the site of inoculation and diffuse infiltration of polymorphonuclear cells and histiocytes. Most of the acid-fast bacilli appeared as clumps in polymorphonuclear neutrophils and in macrophages. Some of these had pyknotic nuclei. By the second day, many of the phagocytic cells containing bacilli had degenerated, leaving clumps of bacilli in ghost cells or free in the tissue but retaining the rounded contours they had formed in the phagocytic cells. Edema of dermal collagen was prominent. By day 5, dermal edema was more pronounced, the inflammatory cells and dermal fibroblasts showed degenerative changes, and there was beginning necrosis of adjacent epidermis above and of the adipose tissue and muscle below. Some of these changes resembled those of the early, human infection (3). Sections prepared 2 days after inoculation of sonically treated supernatant revealed similar changes, including degeneration of neutrophils and macrophages, edema of the dermal collagen, and—to a lesser degree—necrosis of the fat and underlying muscle. Sections at 8 days revealed ulceration (Fig. 5)

with degenerating inflammatory cells and inflammation of the dermis and adipose tissue (Fig. 6, 7). By 30 days there were no obvious changes or evidence of residual scarring. Sections prepared after inoculation of culture filtrate revealed an infiltration of polymorphonuclear cells and histiocytes in the dermis and subcutaneous fat and edema of the dermis. These features were minimal as compared to those from sections prepared after inoculations of the intact organisms or sonically treated supernatant. Furthermore, the reaction subsided within 8 days.

DISCUSSION

Although infection of man by *M. ulcerans* was first described 26 years ago (9), the cause of the necrosis has not been determined. The lesions caused by *M. ulcerans* are characterized histopathologically by extensive coagulation necrosis; this, plus the fact that organisms may be confined to a small zone within the extensive area of necrosis, suggests the effects of a diffusing toxic substance. Although degenerating, inflammatory cells release tissue-damaging enzymes, these enzymes tend to produce suppuration and liquefaction necrosis, features not seen in *M. ulcerans* infections. Furthermore, the extensive coagulation necrosis associated with *M. ulcerans* is, as far as we know, a unique feature of *M. ulcerans* infection, and we believe that the necrosis is the consequence of a unique toxin. To support this, our studies reveal that fractions prepared from cultures of *M. ulcerans* have a toxic effect on mammalian tissue cell cultures and on guinea pig skin.

The Zairian strains of *M. ulcerans* and strain no. 455 from a Nigerian student had recently been isolated and were cytotoxic. These strains also produced gross lesions in the footpads of mice, similar to those described previously (5). All other species of mycobacteria except *M. marinum* failed to produce a CPE. The supernatants of *M. marinum* produced a weaker and qualitatively different CPE, and the toxicity of *M. marinum* is being studied. Culture filtrates from the strains of *M. ulcerans* isolated at 37 C produced varied or unpredictable cytopathogenic effects. The same was true for those strains that produced no ulcer in the mouse footpad and also for those that produced a delayed ulcer, i.e., appearing more than 5 weeks after inoculation. But, those strains of *M. ulcerans* which showed maximal cytopathogenic effect were, without exception, those strains which were isolated at 32 C only and which produced ulcers in the mouse footpad in less than 5 weeks.

The severity of the disease in the patients and its pathogenicity for animals did not appear to have any direct relationship to its CPE for tissue cells. Any attempt at such a correlation should evaluate many factors. As examples of varied responses, one strain (71-10) isolated from a patient with a severe advancing ulcer produced no lesion in the mouse footpad; conversely, strain 71-205 from a patient with a mild infection produced an ulcer in the mouse footpad within 3 weeks. Also, the virulence of the strains varied greatly, as determined by the time of onset of ulcers in the footpads of mice (Table 1).

Animal passage may enhance or restore virulence. For instance, strain 70-22B, when inoculated after isolation, produced ulcers in 18 weeks. After one animal passage, however, it produced ulcers in 5 weeks. Conversely, repeated subcultures of a strain may reduce virulence. The RF strain, although pathogenic for mice when first isolated, failed to produce ulcers after repeated subculture in vitro.

M. marinum did not cause ulceration, but the feet became swollen and red as described previously. The BCG strain produced transient swelling for 2 weeks only. The *Mycobacterium* sp. that culturally resembled *M. tuberculosis* was avirulent for guinea pigs and produced no swelling, redness, or ulceration of footpads. Ulcers developed more quickly when the infecting dose was large.

The variable resistance of individual mice may also be a factor in causing time differences in onset of ulcers. Figure 2 indicates two parameters we used for comparing strains—the death rate of the mice and the progress of the swelling of the feet. These show the variability within a group of animals inoculated at the same time. The time of development of an ulcer was retained, however, as the criterion for comparing the relative virulence of the different strains. Ulceration usually occurred within 2 weeks in all mice after the first animal in the group developed an ulcer. The time of death of individual animals within groups of animals in all studies occurred over several weeks (Fig. 2).

Our preliminary studies indicate that the toxic substance is heat labile and has a molecular weight in the range of the smaller proteins. Further studies are in progress.

ACKNOWLEDGMENT

This investigation was supported in part by a research contract, project number 3A061102B71Q, from the Medical Research and Development Command, U.S. Army, Washington, D.C.

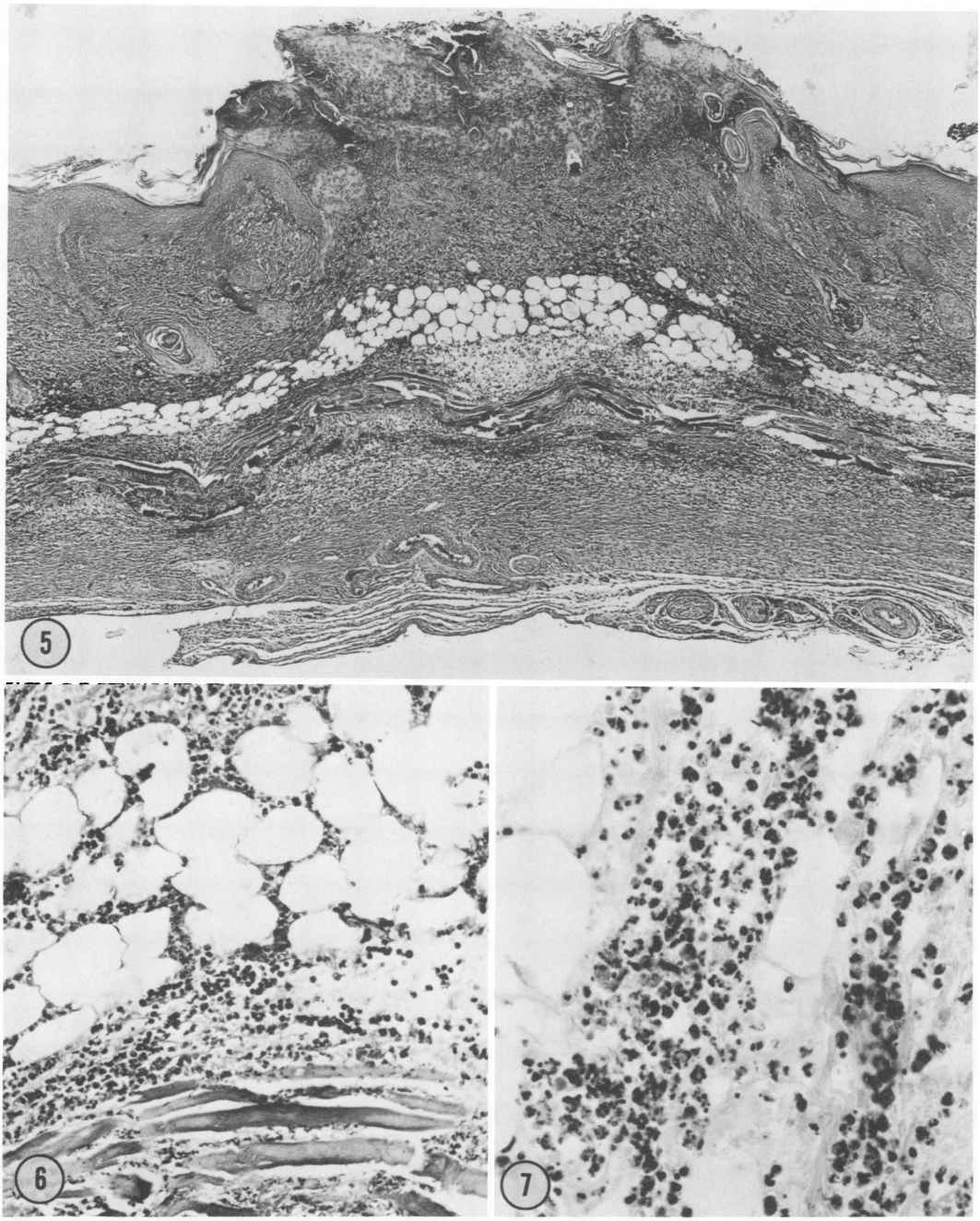

FIG. 5. *Section of guinea pig skin prepared 8 days after intradermal inoculation of sonically treated supernatant. A dry eschar covers the surface, and the epithelium at the margin has begun to proliferate and grow around the inflamed area. The dermis, subdermal adipose tissue, and underlying muscle are edematous and infiltrated with inflammatory cells. Hematoxylin and eosin; ×22; AFIP negative 73-520.*

FIG. 6. *Higher magnification of the adipose tissue in the same slide as Fig. 5. The nuclei of the fat cells and the nuclei between the dermal collagen fibers are gone. The histiocytes and polymorphonuclear cells have infiltrated between and around the adipose tissue cells. Hematoxylin and eosin; ×180; AFIP negative 73-519.*

FIG. 7. *Higher magnification of an area shown in Fig. 6. Many of the histiocytes and polymorphonuclear cells are degenerating. Hematoxylin and eosin; ×290; AFIP negative 73-4675.*

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