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Biochemical and Morphological Characterization of the Killing of Human Monocytes by a Leukotoxin Derived from *Actinobacillus actinomycetemcomitans*

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A potent, heat-labile leukotoxic material was extracted from *Actinobacillus actinomycetemcomitans* (strain Y4), an anaerobic gram-negative microorganism originally isolated from subgingival plaque in a patient with juvenile periodontitis. The cytopathic effects of Y4 toxin on purified monocytes were studied by the extracellular release of radioactive cytoplasmic markers and cell enzymes and by time-lapse microcinematography. Y4 toxin rapidly bound to the cells, producing dose- and time-dependent alterations culminating in cell death and release of intracellular constituents into the culture medium. The evidence to be presented suggests that the cell membrane of the monocyte may be the primary target in the development of these phenomena. Previous studies have shown that Y4 toxin also kills human polymorphonuclear leukocytes but not other cell types. It is conceivable that disruption of polymorphonuclear leukocytes and monocytes by Y4 toxin in the gingival crevice area may be relevant in the pathogenesis of juvenile periodontitis.

In a series of in vitro studies, we have shown that dental plaque (44) as well as a variety of viable gram-positive (42, 43) or gram-negative (46) plaque isolates can trigger extracellular release of lysosomal enzymes from human or rabbit polymorphonuclear leukocytes (PMNs). Page and his colleagues have also demonstrated that mouse peritoneal macrophages discharge lysosomal markers into the culture medium when incubated with homogenates of dental plaque (28) or various plaque-derived microorganisms (29). Under these conditions, lysosomal enzyme release from PMNs or macrophages was an active secretory process as opposed to a secondary consequence of cell death. Thus, stimulated cells showed no obvious morphological signs of injury, were not stained by trypan blue, and did not release the cytosolic enzyme lactate dehydrogenase (LDH) into the surrounding medium.

We have recently found that *Actinobacillus actinomycetemcomitans* (strain Y4), a gram-negative, anaerobic plaque isolate, caused lysosomal enzyme release accompanied by killing of PMNs (1). This organism was originally cultured from a sample of subgingival plaque of a patient with juvenile periodontitis and caused periodontal lesions upon oral inoculation in gnotobiotic rats (14). A soluble, macromolecular, heat-labile, protease-sensitive toxin was extracted from Y4 by various procedures (45). This material destroyed isolated human PMNs as judged by electron microscopy and by LDH release. On the basis of dye exclusion experiments, Y4 toxin also had toxic effects on human monocytes. However, the toxin did not kill human lymphocytes, fibroblasts, platelets, or erythrocytes, nor did it adversely affect rabbit, rat, or murine PMNs (45). Although the role of Y4 in the pathogenesis of juvenile periodontitis has not been established, it seems reasonable to speculate that adverse effects of the toxin on PMNs and macrophages may be pertinent to the development of this disorder. Death of these cells may compromise antibacterial host defense in the periodontal area. In addition, the consequent release of lysosomal enzymes from such cells could have deleterious effects on surrounding connective tissue elements. In view of these considerations, we have extended our work to characterize the in vitro effects of Y4 toxin on human monocytes.

MATERIALS AND METHODS

Reagents and chemicals. Penicillin, streptomycin, zymosan A (from *Saccharomyces cerevisiae* yeast), bovine serum albumin (Cohn fraction V), α-methyl-D-mannoside, indomethacin, sodium fluoride, sodium azide, 2-deoxy-D-glucose, iodoacetic acid, colchicine, chloroquine, cycloheximide, cytochalasin B, phenylglyoxal, and ammonium acetate were obtained from Sigma Chemical Co., St. Louis, Mo. Heparin,

preservative free, was from Paines & Byrne, Greenford, England; pyruvate and reduced nicotinamide adenine dinucleotide were from Boehringer Mannhein GmbH, West Germany; Triton X-100, D(+)-glucose, D(+)-galactose, sucrose, and ethylenediaminetetraacetic acid (disodium salt, EDTA) were from British Drug House Chemicals Ltd., Poole, England; D(+)-mannose was from Hopkins and Williams Ltd., Chadwell Heath, England; neuraminidase (*Clostridium perfringens*) and trypsin (bovine pancreas) were from Worthington Biochemical Corp., Freehold, N.J.; D(+)-fucose, *p*-nitrophenyl-2-acetamido-2-β-D-glucopyranoside, and *N*-acetyl-glucosamine were from Koch Light Co., Colnbrook, England; dextran T500 and Ficoll-Paque were from Pharmacia, Uppsala, Sweden; RPMI-1640 and fetal calf serum were from GIBCO, Paisley, Scotland; polystyrene latex particles (0.81-μm diameter; 5%, vol/vol) and *Escherichia coli* lipopolysaccharide (O111:B4) were from Difco Laboratories, Detroit, Mich.; insulin was from Wellcome Reagents Ltd., London, England; dexamethasone (sodium phosphate) was from Organon Laboratories, Morden, England; chrysotile asbestos (UICC Standard reference sample of Rhodesian chrysotile), sodium ^{51}chromate (^{51}Cr, 100 to 400 mCi/mg), and ^{86}rubidium chloride (^{86}Rb, 6.9 mCi/mg) were from Radiochemical Centre, Amersham, England.

Extraction of Y4 toxin. A sonic extract of Y4 was prepared as described by Tsai et al. (45). Briefly, Y4 was grown in thioglycolate broth under anaerobic conditions and harvested at 48 h. Washed cells were suspended in distilled water and subjected to sonication for 15 min at 2°C. The supernatant obtained by centrifugation was dialyzed against distilled water and lyophilized. The lyophilizate was redissolved (1.0 mg [dry weight] per ml) in phosphate-buffered saline (136 mM NaCl, 2.7 mM HCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, without Ca^{2+} or Mg^{2+}), and 1.0-ml portions were stored at −20°C until use. A single such pool of Y4 toxin was employed in the experiments and contained 530 μg of protein per ml as determined by the Lowry procedure, with bovine serum albumin as the standard (19). This toxin preparation was able to kill human PMNs but had no demonstrable lytic effects on human lymphoid cells or erythrocytes (45).

Isolation and cultivation of human monocytes. Heparinized (5 U/ml) buffy coat residues were secured from healthy donors and sedimented by gravity in the presence of equal volumes of 2% dextran for 45 min at room temperature. The leukocyte-enriched supernatant was centrifuged (400 × *g*, 30 min, 20°C) on Ficoll-Paque gradient (3). The mononuclear cell interface was washed twice by centrifugation (400 × *g*, 4 min, room temperature) in phosphate-buffered saline and twice in warmed RPMI-1640 buffered with sodium bicarbonate. Cell counts were adjusted to 2.0×10^6 leukocytes per ml.

Cultivation of the cells was based on the method of Hovi et al. (13). A 3-ml amount of the cell suspension was added to each well of a tissue culture cluster (Costar, 3506, Cambridge, Mass.) and allowed to adhere (in the absence of serum) at 37°C for 45 to 60 min in an incubator gassed with 5% CO_2 (95% air). Cultures were then washed four times in warmed Hanks balanced salt solution to remove nonadherent cells and incubated for 10 to 12 h in serum-free RPMI-1640 buffered with sodium bicarbonate and containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). Subsequently, the medium was replaced with RPMI-1640 supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum, which was employed thereafter unless otherwise indicated. A single pool of fetal calf serum was used in the experiments. After a period of 4 days, the cultures were washed two times in warmed Hanks balanced salt solution and maintained in RPMI-1640 supplemented with fetal calf serum, which was replaced every 48 h. Cells were exposed to Y4 toxin between 6 and 10 days after isolation, and at this time the adherent cell population consisted of large, extended cells containing numerous phase-dense cytoplasmic granules (Fig. 1, zero time). Electron microscopic examination of representative cultures revealed that the cells had the morphological characteristics of macrophages whose cytoplasm contained numerous granules, vacuoles, and lipid-like inclusions. At least 95% of the cells were capable of ingesting latex spherules (2 μl/ml) or zymosan A particulates (50 μg/ml) which were added to the cultures for periods of 12 to 18 h. Although varying numbers of lymphoid cells and platelets were visible during the early stages of culture, these elements were not apparent after the washing procedure in the 4-day cultures.

Exposure of monocytes in Y4 toxin. The medium was withdrawn from the cultures and replaced with 3.0 ml of RPMI-1640 (serum free) before the addition of different concentrations of Y4 toxin (in a total volume of 0.3 ml of phosphate-buffered saline) for varying periods of time. In standard experiments this consisted of a 3-h incubation in the presence of 10 μg of toxin per ml. All incubations were carried out in triplicate. At the conclusion of the experiment the medium was withdrawn from the cultures, and the remaining adherent cells were scraped from the dish in 0.1% Triton X-100 (for enzyme assays) or in 1.0% Triton X-100 (for radiolabel assays).

Assessment of monocyte response to Y4 toxin.
(i) Morphological studies. Cultures were monitored with conventional and phase-contrast optics at varying intervals (up to 4 h) after addition of Y4 toxin. Time-lapse microcinematography was carried out as described previously (34) with monocytes cultured on glass cover slips which were placed into Sykes-Moor Chambers (Bellco Glass Co., Vineland, N.J.). Toxin was introduced into the chambers, and films were made at the rate of one frame every 3 s with an exposure of 0.2 s. Cell death was monitored with both phase-contrast and Nomarsky differential-contrast optics with a ×40 objective.

(ii) Release of ^{51}Cr or ^{86}Rb from monocytes. Cultures were double labeled with 5.0 μCi of ^{51}Cr sodium and 5.0 μCi of ^{86}Rb chloride for 60 min at 37°C in a total volume of 1 ml of RPMI-1640 (serum free) (33). Excess isotopes were removed by four washes with Hanks balanced salt solution, and the cells were then exposed to Y4 toxin as described previously. By using gamma spectroscopy, the percentage of ^{51}Cr or ^{86}Rb released extracellularly from the cells at different times was calculated from activities measured in the culture medium and in the remaining adherent cells lysed with Triton X-100 (1.0%).

(iii) Enzyme release from monocytes. The procedures employed to assay various enzymes have been detailed previously (8). Briefly, LDH was measured by the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm. *N*-acetyl-β-D-glucosaminidase (N-Ac-Glu) was assayed with *p*-nitrophenyl-2-acetamido-2-β-D-glucopyranoside dissolved in 0.1 M citrate phosphate buffer (pH 4.5) as substrate. β-Glucuronidase was estimated in representative cultures. Enzyme release was expressed as a percentage of the total activity in the cultures (i.e., culture supernate plus cell lysate). Inherent enzyme activities in any reagents (e.g., serum) were taken into account in all calculations.

Effects of various pharmacological agents on Y4-mediated killing of monocytes. Several pharmacological agents were tested to ascertain whether they could modulate cell lysis in these experiments. Unless otherwise stated, several concentrations of these compounds were preincubated with monocytes for 60 min at 37°C in RPMI-1640 (serum-free). Y4 toxin was added to the cultures for an additional 3-h period. The vast majority of drugs failed to inhibit cell lysis. Data obtained with the highest concentration of the test agent which demonstrated no significant cytopathic effects of its own are used for illustrative purposes in most instances.

Additional experiments are described below.

RESULTS

Morphological changes in monocytes exposed to Y4 toxin. When observed by time-lapse cinematography, different cells showed fairly consistent morphological changes, although the time course of changes varied considerably from one cell to another. Figure 1 illustrates the alterations in two adjacent cells. Based upon observations on several cultures, the changes could be divided into three phases. Phase 1 lasted 5 to 10 min and was characterized by cessation of the normal undulating movements of the membrane folds, accompanied by withdrawal of the cytoplasm towards the center of the cell. Under Nomarski optics, the cell seemed to flatten onto the glass, so that the area over the nucleus appeared as a distinct bulge (not illustrated). Phase 2 spanned a period of variable duration (5 to 60 min) in which the cell remained quiescent except for abnormal movement of the membrane and the formation of strings of cytoplasm projecting from the cell. In some cases cytoplasmic granules appeared more obvious, and the nucleus became more distinctly demarcated (this nuclear change was most clearly seen with Nomarski optics). Phase 3 consisted of an explosive release of cytoplasmic material from the cells over a period of less than 1 min. In some cells phase 3 commenced as early as 5 min after exposure to Y4 toxin, whereas in others phase 3 was delayed for up to 60 min. Phase 3 was terminated by ballooning of the cells which were grossly swollen and obviously dead at this point.

Dose dependence and time course of monocyte lysis in response to Y4 toxin. As reflected by release of LDH into the culture medium, cell lysis was a dose-dependent phenomenon (Fig. 2). During a 3-h exposure in the absence of serum, 10 μg of toxin per ml induced significant extracellular release of LDH, reaching a plateau at approximately 50 μg/ml (Fig. 2). N-Ac-Glu release paralleled but was substantially less than LDH release (Fig. 2). The inclusion of 10% fetal calf serum in the medium during exposure to Y4 toxin did not affect the magnitude of the release response (Fig. 2). Toxin which had been heated (56°C, 30 min) failed to lyse monocytes in the presence or absence of fetal calf serum (Fig. 2). The fact that the toxin was heat-labile indicates that it is not bacterial lipopolysaccharide.

Release of intracellular markers into the culture medium was also a function of the duration of exposure to Y4 toxin. By using a constant dose of toxin (10 μg/ml) in serum-free RPMI-1640, it is apparent that within 5 min the release of the potassium analog ^{86}Rb was substantially higher than controls. (Fig. 3). ^{86}Rb release was virtually complete by 15 min. ^{51}Cr release commenced between 15 and 30 min and continued in a linear fashion, reaching a maximum of approximately 60% by 60 min (Fig. 3). LDH release was detectable from 30 min, reaching a maximum of about 60% at 120 min (Fig. 4). N-Ac-Glu (Fig. 5) and β-glucuronidase (data not shown) release had similar profiles but were not as extensive as LDH release. In comparing the kinetics of extracellular enzyme release (Fig. 4 and 5), the data suggest that N-Ac-Glu release may occur more rapidly than LDH release.

On the basis of the preceding data, subsequent experiments with Y4 toxin were carried out under the standard conditions defined in Materials and Methods, namely, exposure of cells to 10 μg of toxin per ml for a period of 3 h. In a series of pilot studies (data not presented), various concentrations (10 to 100 μg/ml) of sonic extracts from strains ATCC 29522 and 29524 but not ATCC 29523 of *A. actinomycetemcomitans* produced toxic effects on human monocytes as described for Y4 toxin. Contrariwise, sonic extracts from *Bacteroides melaninogenicus* (strain 381) or *Actinomyces viscosus* (T14, virulent strain) did not kill human monocytes.

Association of Y4 toxin with monocytes. Monocytes were exposed to Y4 toxin (10 μg/ml) for 1 to 15 min at 37°C. Subsequently the cultures were washed four times in chilled Hanks balanced salt solution to remove unbound toxin and then incubated at 37°C for an additional 3

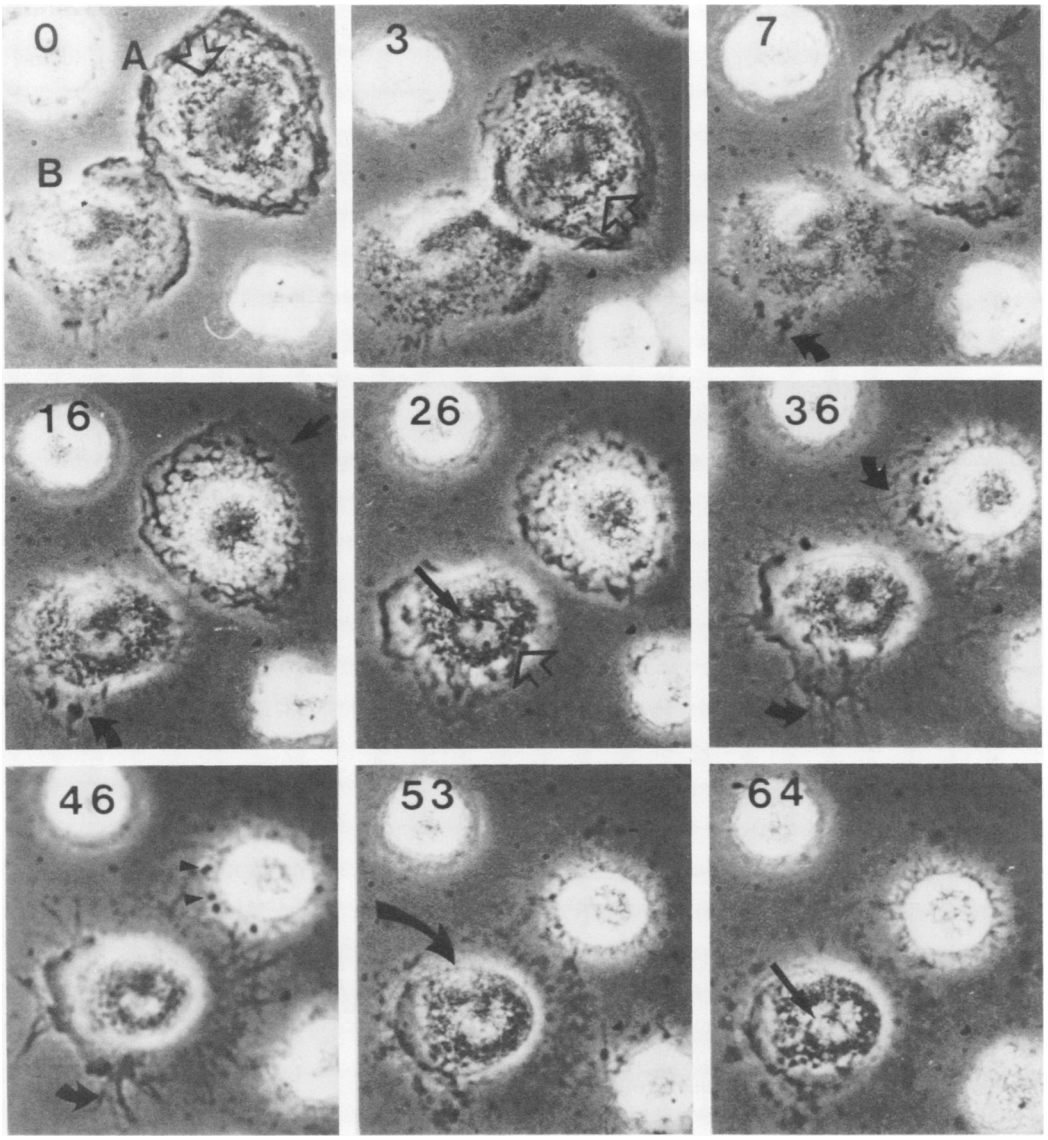

FIG. 1. *Sequence of photographs taken from a lapse film of monocytes exposed to Y4 toxin. Time in minutes after the addition of Y4 toxin is given on each frame. Zero time is taken from a control sequence filmed before adding toxin to the culture. Monocytes A and B demonstrate the characteristic features of cultured monocytes; note the peripheral folds of the membrane and the presence of numerous cytoplasmic granules (open arrow). Two smaller monocytes are also seen but are not labeled; they are normal, viable cells which have not spread out on the cover slip. The response of cells A and B to Y4 toxin will be described individually. Cell A: Within 2 min after exposure to Y4 toxin, there was a cessation of the undulating movement of the membrane folds. The cytoplasm began to retract, causing an accumulation of granules in the perinuclear area (open arrow at 3 min). As cytoplasmic retraction continued, the outline of the adhering cell membrane was still visible (7 and 16 min). The cell became progressively "piled up" so that it appeared opaque (or white) under phase contrast. Strands of material projected from the cell (short curved arrow at 36 min). At approximately 46 min, the cytoplasmic granules were ejected from the cell (arrowheads). No further morphological changes were seen up to 64 min; thus, lysis of this cell is incomplete up to this point. Cell B, Cell membrane retraction was evident during 3 to 6 min after exposure to Y4 toxin. This was followed by slight "bubbling" of the membrane (curved arrow at 7 and 16 min). The nucleus became shrunken and more distinctly outlined (straight arrow at 26 min), whereas the cytoplasmic granules assumed a perinuclear distribution (open arrow at 26 min). An explosion of fine particles from the cell was visible in the film at 25 min, but this was not discernible in the still reproductions. Strands of material projected from the cell (short curved arrow at 36 and 46 min). By 36 min all cell movement had ceased. At 53 min there was a sudden ballooning of the cell (long curved arrow at 53 min), leaving an almost empty cell carcass, with shrunken remnants of the nucleus still visible (straight arrow at 64 min). Magnification, ×660.*

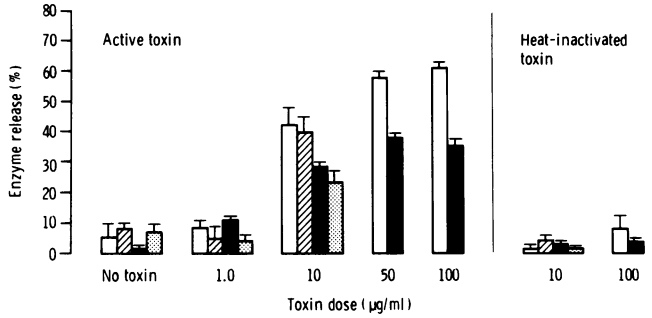

FIG. 2. *Dose-dependence of monocyte response to Y4 toxin. Monocytes were incubated with varying concentrations of active (nonheated) and heat-inactivated (56°C, 30 min) Y4 toxin for 3 h at 37°C. LDH release (± standard deviation) in the absence (□) or presence of (▨) of 10% (vol/vol) fetal calf serum. N-Ac-Glu release in the absence (■) or presence (▩) of the fetal calf serum. Values represent means (± standard deviation).*

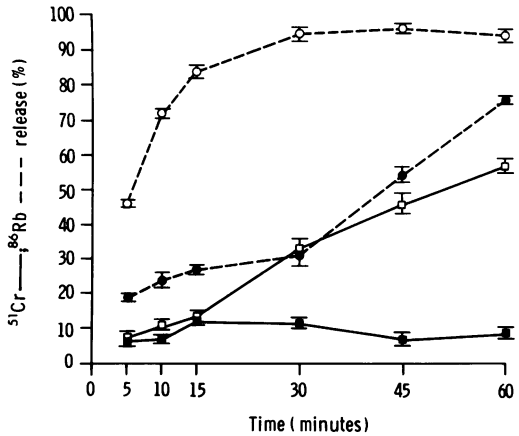

FIG. 3. *Release of ^{86}Rb and ^{51}Cr from monocytes exposed to Y4 toxin (10 μg/ml) for varying time periods at 37°C in serum-free medium. ^{86}Rb release (- - -) from control (●) and toxin-treated (○) cultures. ^{51}Cr release (——) from control (■) and toxin-treated (□) cultures. Values represent mean (± standard deviation).*

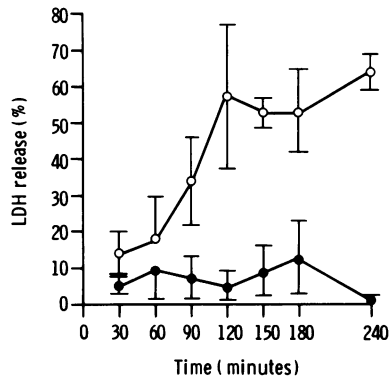

FIG. 4. *Release (mean ± standard deviation) of LDH from monocytes exposed to Y4 toxin (10 μg/ml) for varying time periods at 37°C in serum-free medium. Symbols: ●, control cultures; ○, cells exposed to toxin.*

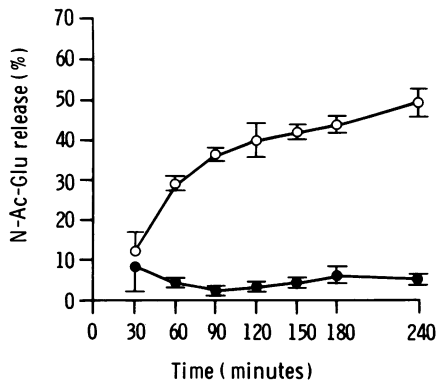

FIG. 5. *Release (mean ± standard deviation) of N-Ac-Glu from monocytes incubated with Y4 toxin (10 μg/ml) for varying time periods at 37°C. Symbols: ●, control cultures; ○, cells exposed to toxin.*

h in RPMI-1640 (without toxin). Within 5 min of exposure, sufficient toxin had become firmly associated with the cells to mediate significant LDH and N-Ac-Glu release during the subsequent 3 h of incubation (Fig. 6). It is possible, but not proven, that the degree of enzyme release paralleled the amount of toxin associating with the cells during the varied preincubation periods.

A crude membrane fraction of human monocytes was prepared by repeated freeze-thawing of cell suspensions in phosphate-buffered saline (10×10^6 cells per ml): 0.4 ml of the membrane fraction was preincubated with varying concentrations of Y4 toxin (90 min, 4°C). The mixture was then centrifuged to remove insoluble partic-

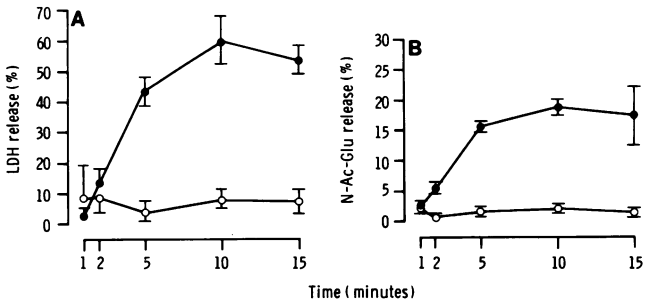

FIG. 6. *Association of Y4 toxin for monocytes. As described in the text, monocytes were incubated with Y4 toxin (10 μg/ml) at 37°C for 1 to 15 min. Cultures were washed to remove unbound toxin and then incubated for an additional 3 h at 37°C. Release (mean ± standard deviation) of LDH (A) and N-Ac-Glu (B) was measured in control* (○) *and experimental* (●) *cultures at the conclusion of the 3-h period.*

ulates, and the supernatant was tested for residual toxic activity. Under these conditions, monocyte membranes removed 65% of the toxic activity from 40 μg of Y4 sonic extract per ml, and over 80% of the toxic activity from either 10 or 20 μg of Y4 sonic extract per ml. PMNs but not human erythrocyte membranes prepared in an identical fashion were also capable of absorbing out toxic activity from Y4 sonic extracts.

Lysis of monocytes by Y4 toxin at 4°C. Cells were incubated at 4°C for 60 min in a gas-sealed (5% CO_2–95% air) environment before addition of toxin to the cultures for varying periods of time at 4°C. When compared with cells exposed to toxin at 37°C (Fig. 3 and 4), it was apparent that monocytes still discharged significant amounts of enzymes after exposure to toxin at 4°C (Fig. 7). Under these conditions, LDH release was retarded initially, but by 20 h, when the cells appeared to be grossly swollen, approximately 50% of the available LDH could be assayed in the culture medium (Fig. 7). Likewise, N-Ac-Glu discharge was initially delayed at 4°C, but by 20 h approximately 25% could be measured extracellularly (Fig. 7). In comparing enzyme release profiles at 4°C, it is evident that by 8 h N-Ac-Glu release from toxin-treated cells appeared to be significantly greater than that released spontaneously from control cells. At this time there was no difference in the amount of LDH liberated from control versus experimental cultures. In other words, N-Ac-Glu release preceded LDH release under these conditions.

Lysis of monocytes in the presence of osmotic protectants. Monocytes which were preincubated (60 min, 37°C) with varying concentrations (1 to 10%) of bovine serum albumin released significantly less LDH and N-Ac-Glu in response to Y4 toxin (Table 1) and were not swollen by the end of the experiment. Similarly, cells preincubated in RPMI-1640 containing

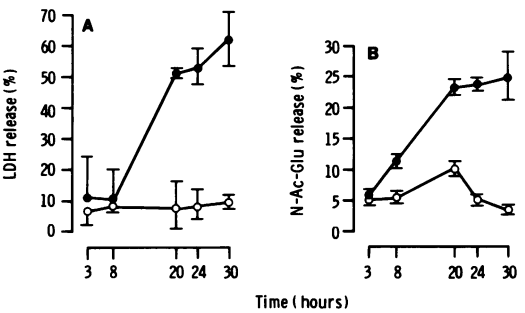

FIG. 7. *Lysis of monocytes in response to Y4 toxin at 4°C. Monocytes were incubated with toxin (10 μg/ml) for varying periods of time at 4°C, and release (mean ± standard deviation) of LDH (A) and N-Ac-Glu (B) was assayed in control* (○) *and experimental* (●) *cultures.*

1.0% dextran were not swollen by the toxin as monitored by phase-contrast microscopy.

Lysis of monocytes in the presence of cytochalasin B. Monocytes which had been preincubated with cytochalasin B (5 μg/ml) for 60 min were still susceptible to lysis after exposure to Y4 toxin (Table 1). Cytochalasin B is a fungal metabolite which disrupts microfilaments causing an associated loss of cell functions dependent on these structures (e.g., phagocytosis, chemotaxis, etc.) (20, 31). This compound may also interfere with the Fc receptor on human monocytes (49).

Effects of agents which modify the cell surface. Monocytes were treated with several agents known to modify various membrane functions. Cells were preincubated (60 min, 37°C) with phenylglyoxal (10^{-4} to 10^{-6} M) (40) or insulin (10 to 100 mg/ml) (23, 38) before adding Y4 toxin to the medium. Trypsin (10 to 100 μg/ml) (47) or neuraminidase (0.1 to 0.5 U/ml) (16) was added to monocytes for 60 min at 37°C; cells were then washed four times in RPMI-1640 be-

fore exposure to Y4 toxin. None of these agents prevented toxin-mediated lysis (Table 1). In fact, phenylglyoxal and insulin appeared to accentuate LDH release. 2-Deoxy-D-glucose, which inhibits Fc and complement receptor-mediated phagocytosis in mouse macrophages (21), was also ineffective in preventing cell lysis (see below, Table 3).

Effect of heparin and various sugars on cell lysis. A variety of simple sugars have been shown to interfere with binding between bacteria and phagocytic cells (2, 11). We wanted to determine whether similar agents could impair lysis in response to Y4 toxin. Several sugars as well as heparin were preincubated (60 min, 37°C) with monocytes followed by the addition of toxin to the cultures. Heparin-treated cells did not become swollen when incubated with Y4 toxin, nor did they release enzymes into the culture medium (Table 2). In the presence of α-methyl-D-mannoside there was some protection against Y4 toxin, but none of the other sugars affected the degree of cell injury (Table 2).

Effects of agents which modify cell metabolism. Monocytes were preincubated (60 min, 37°C) with compounds known to alter various metabolic functions in these cells (7, 11, 22). Toxin was subsequently added directly to the cultures. None of the agents tested inhibited cell lysis (Table 3). Sodium fluoride, sodium azide, and a combination of sodium azide and 2-deoxy-D-glucose appeared to amplify LDH release. LDH release was also enhanced in EDTA-treated cultures, but on its own EDTA had significant toxic effects on the cells as reflected by high background release of LDH in control cultures. It is interesting to note that EDTA apparently inhibited the release of N-Ac-Glu in response to Y4 toxin. A similar inhibition of lysosomal enzyme release had been observed when PMNs are exposed to the toxin in the presence of EDTA (45). Since divalent cations are prerequisite for membrane fusion (30), it is possible that EDTA limited lysosomal enzyme release by restricting the degranulation observed at the beginning of phase 3 in the cytotoxic process (see above).

Effect of anti-inflammatory agents. Var-

TABLE 1. *Effect of various agents on release of enzymes from monocytes exposed to Y4 toxin*[a]

Condition	Enzyme release (%)			
	LDH		N-Ac-Glu	
	Control	Y4 toxin	Control	Y4 toxin
Standard experiment	5.7 ± 5.9	49.9 ± 8.2	4.1 ± 2.3	32.3 ± 5.9
Bovine serum albumin (5%, wt/vol)	6.9 ± 4.4	27.9 ± 3.0	6.8 ± 1.0	23.0 ± 1.9
Trypsin (10 μg/ml)	2.7 ± 2.3	46.9 ± 12.5	5.6 ± 1.0	34.8 ± 3.2
Neuraminidase (0.1 U/ml)	6.1 ± 5.6	64.5 ± 17.5	3.2 ± 1.2	33.3 ± 2.2
Insulin (50 ng/ml)	4.8 ± 4.3	66.0 ± 6.5	3.2 ± 1.0	44.5 ± 8.1
Phenylglyoxal (10^{-6} M)	6.9 ± 8.2	83.6 ± 9.6	5.8 ± 3.3	34.3 ± 3.5
Cytochalasin B (5 μg/ml)	2.2 ± 3.8	56.1 ± 1.0	4.9 ± 1.0	33.3 ± 2.1

[a] Monocytes were pretreated with various agents as described in the text and subsequently exposed to Y4 toxin (10 μg/ml) for a period of 3 h. Results represent mean (± standard deviation).

TABLE 2. *Effect of heparin and various sugars on cytotoxic release of enzymes from monocytes exposed to Y4 toxin*[a]

Condition	Enzyme release (%)			
	LDH		N-Ac-Glu	
	Control	Y4 toxin	Control	Y4 toxin
Standard experiment	3.6 ± 4.6	48.9 ± 9.8	5.7 ± 4.5	31.9 ± 5.9
Heparin (10 U/ml)	0	4.1 ± 7.1	1.1 ± 0.3	4.6 ± 0.1
α-Methyl-D-mannoside (10^{-4} M)	2.6 ± 2.5	30.0 ± 12.5	2.6 ± 0	22.0 ± 1.3
α-Methyl-D-mannoside (10^{-5} M)	1.3 ± 2.2	34.9 ± 3.5	2.2 ± 0	23.5 ± 1.8
Galactose (10^{-5} M)	1.1 ± 1.1	40.1 ± 5.1	6.9 ± 2.9	34.3 ± 3.5
Glucose (10^{-5} M)	4.1 ± 0	56.1 ± 5.5	10.2 ± 1.0	36.0 ± 1.5
N-acetyl-glucosamine (10^{-5} M)	3.4 ± 1.7	46.4 ± 7.1	7.3 ± 1.8	36.3 ± 6.4
Fucose (10^{-5} M)	7.1 ± 1.0	56.6 ± 2.0	10.2 ± 2.7	46.2 ± 7.4
Sucrose (10^{-5} M)	4.7 ± 1.2	51.3 ± 1.0	7.9 ± 0.5	36.6 ± 1.3
Mannose (10^{-5} M)	0	64.2 ± 4.7	4.4 ± 1.0	30.5 ± 0

[a] Cells were preincubated with various agents as described in the text before the addition of Y4 toxin to the cultures. Results represent mean (± standard deviation).

ious anti-inflammatory compounds are known to affect diverse parameters of monocyte-macrophage activity. In the present study, compounds which may alter prostaglandin or lysosomal enzyme synthesis and release were used to determine whether they could alter the cytotoxic phenomenon. These included: indomethacin (10^{-4} to 10^{-6} M), dexamethasone (10^{-4} to 10^{-6} M), colchicine (10^{-4} to 10^{-6} M), chloroquine (2.5×10^{-6} M), and ammonium acetate (5×10^{-2} M) (4, 9, 17, 32). Dexamethasone was incubated with monocytes for 20 h in RPMI-1640 containing 10% fetal calf serum; the medium was then replaced with serum-free RPMI-1640 plus dexamethasone and Y4 toxin. The other drugs were preincubated with cells for 60 min before adding toxin to the medium. None of these agents afforded demonstrable protection against Y4 toxin (Table 4).

Effects of agents which stimulate macrophages. Upon appropriate treatment, monocytes undergo activation of various cellular and metabolic processes and respond differently to certain stimuli when compared with unactivated cells (24). The monocyte infiltrate in inflamed gingival tissues may represent an activated population of cells (27); therefore, we wanted to determine the susceptibility of preactivated monocytes to Y4 toxin. Accordingly, monocyte cultures were preincubated for 18 h with various particles and soluble substances known to activate macrophages in vitro, including latex spherules (2 μl/ml), zymosan A (50 μg/ml), asbestos fibers (50 μg/ml), and endotoxin (10 μg/ml). The cells were then washed with RPMI-1640 and exposed to Y4 toxin for 3 h. These cells were still susceptible to lysis mediated by the toxin (Table 5).

DISCUSSION

When exposed to Y4 toxin in vitro, human monocytes undergo morphological and biochem-

TABLE 5. *Effect of agents which stimulate monocytes on cytotoxic response to Y4 toxin*[a]

Monocytes preincubated (18 h) with:	Enzyme release (%)			
	LDH		N-Ac-Glu	
	Control	Y4 toxin	Control	Y4 toxin
Medium alone	8.6 ± 9.2	32.1 ± 5.5	2.0 ± 1.0	15.9 ± 1.3
Y4 toxin heat inactivated	5.4 ± 1.2	28.1 ± 12.9	1.5 ± 1.0	20.8 ± 5.6
E. coli endotoxin	2.8 ± 4.9	40.5 ± 6.4	1.0 ± 0	22.8 ± 1.0
Latex particles	3.6 ± 3.1	29.3 ± 5.4	1.0 ± 1.0	10.9 ± 2.0
Zymosan	5.8 ± 4.0	33.0 ± 11.0	2.7 ± 1.0	17.8 ± 3.4
Asbestos	3.7 ± 6.4	29.3 ± 1.6	2.7 ± 1.0	19.0 ± 1.0

[a] Monocytes were preincubated with various stimulants as described in the text and then exposed to Y4 toxin (10 μg/ml) for 3 h.

TABLE 3. *Effect of antimetabolic compounds on enzyme release from monocytes exposed to Y4 toxin*[a]

Condition	Enzyme release (%)			
	LDH		N-Ac-Glu	
	Control	Y4 toxin	Control	Y4 toxin
Standard experiment	5.4 ± 4.7	53.6 ± 10.2	0.9 ± 0.8	29.2 ± 2.5
EDTA (10^{-2} M)	25.4 ± 2.9	71.5 ± 23.4	7.9 ± 1.0	12.1 ± 1.7
Cycloheximide (7×10^{-5} M)	0	51.7 ± 6.6	ND[b]	ND
Sodium fluoride (10^{-7} M)	8.6 ± 4.9	74.8 ± 7.4	10.9 ± 0.1	45.6 ± 1.2
Sodium azide (10^{-2} M)	3.4 ± 4.8	80.8 ± 4.7	8.9 ± 2.1	48.4 ± 5.7
2-Deoxy-D-glucose (2×10^{-2} M)	3.6 ± 3.1	59.2 ± 4.9	6.4 ± 3.7	30.6 ± 5.4
2-Deoxy-D-glucose–sodium azide	6.2 ± 5.8	78.3 ± 4.6	18.4 ± 4.1	64.5 ± 3.5

[a] Same as in Table 2.
[b] ND, Not determined.

TABLE 4. *Effect of anti-inflammatory drugs on enzyme release from monocytes exposed to Y4 toxin*[a]

Condition	Enzyme release			
	LDH		N-Ac-Glu	
	Control	Y4 toxin	Control	Y4 toxin
Standard experiment	4.6 ± 5.1	57.3 ± 8.7	2.7 ± 3.0	29.8 ± 3.8
Indomethacin (10^{-5} M)	10.0 ± 14.1	53.6 ± 5.0	7.4 ± 2.1	35.6 ± 5.7
Colchicine (10^{-5} M)	11.1 ± 11.1	56.6 ± 6.7	5.4 ± 0.8	32.6 ± 2.1
Dexamethasone (10^{-6} M)	1.6 ± 2.7	52.3 ± 3.2	4.4 ± 2.2	26.8 ± 4.0
Chloroquine (2.5×10^{-5} M)	4.4 ± 4.4	67.5 ± 5.6	10.8 ± 3.9	47.9 ± 1.6
Ammonium acetate (5×10^{-2} M)	0	69.4 ± 7.2	10.1 ± 4.5	48.1 ± 5.1

[a] Same as in Table 2.

ical changes indicative of cell death. Similarly, human PMNs but not other cell types suffer irreversible insult when incubated either with whole Y4 microorganisms (1) or with Y4 toxin (45). The results of the present investigation lead us to hypothesize that at least three stages are involved in the lethal response of monocytes to Y4 toxin. First, the toxin probably binds to the cell membrane of the monocyte. This occurs within the first 5 min during which there is a cessation of normal membrane movement and apparent shrinkage of the cytoplasm. Release of the potassium analog ^{86}Rb commences at this time and is virtually completed by 15 min. Such perturbations also seem to be one of the earliest indicators of cell damage in response to other leukocidins derived from staphylococci (48) and from *Pseudomonas aeruginosa* (36). Second, a lag phase ranging from 5 to 60 min follows during which no appreciable extracellular leakage of cytoplasmic markers occurs. There is considerable variation in the length of this second phase with different individual cells, even under identical conditions, as for example in the same field in the microscope. During the second phase there is no cell movement apart from slight abnormal ruffling of the membrane, and the formation of cytoplasmic threads projecting from the cell. At this time the cell remains morphologically intact, although considerably altered in appearance. The third phase is associated with the sudden release of cytoplasmic contents from dying cells. This can be monitored by time-lapse cinematography and by elevated levels of ^{51}Cr, LDH, and lysosomal enzymes in the culture medium. It is likely that the linear release of these cytoplasmic constituents reflects variations in the duration of phase 2 for individual cells in the cultures. It should be noted that ^{51}Cr is not released during phase 1 of the response to the toxin at a time when considerable ^{86}Rb is leaving the cells. This suggests that the increase in permeability during phase 1 may be restricted to the Na/K system. Although LDH release from injured cells can be inhibited by albumin or high-molecular-weight dextran, the mechanism of cell death is probably not primarily a result of osmotic lysis because swelling occurs after the explosive release of cytoplasmic contents. This view is strengthened by ultrastructural observations of PMNs exposed to Y4 toxin in the presence of bovine serum albumin (45). Such PMNs failed to release LDH but demonstrated obvious morphological alterations, suggesting that profound cytopathic changes had occurred. As pointed out by Seeman (39) a decrease in the extracellular release of LDH from dying cells in albumin-enriched medium may be due to a reduction in the solubility and diffusibility of the enzyme.

The concept that Y4 toxin probably binds to the cell membrane is supported by the finding that crude membrane preparations of monocytes were able to remove toxic activity from Y4 sonic extracts. Furthermore, the toxin became firmly associated with the cells within 5 min of exposure at 37°C. When such cells were washed free of unbound toxin, they underwent lysis, discharging LDH and N-Ac-Glu into the culture medium. In similar experiments conducted at 4°C instead of 37°C (data not reported), monocytes absorbed sufficient toxin to be lysed upon rewarming to 37°C. Although delayed initially, cell death also occurred when cells were incubated with toxin for several hours at 4°C rather than 37°C. The toxin also killed monocytes in the presence of cytochalasin B. These conditions (4°C and cytochalasin B) suppress endocytosis to varying degrees (40), and the results suggest that internalization of the toxin may not be a prerequisite for cell killing. However, it is necessary to point out that even under these circumstances small amounts of toxin might still enter the cells by micropinocytosis to affect intracellular targets (see discussion by Gill [12]). α-Methyl-D-mannoside partially inhibited cytotoxicity, presumably by interfering with binding of the toxin to carbohydrate moieties on the cell membrane. Other workers have found that simple sugars can prevent interaction of bacteria (2, 11) or toxins (12) with mammalian cells. Heparin completely inhibited extracellular release of enzymes from cells exposed to Y4 toxin. This anionic polyelectrolyte has been shown to block "binding" of lipopolysaccharides to erythrocytes (10). It is conceivable that an analogous phenomenon is relevant in our system. In similar studies with PMNs instead of monocytes as the target, it has been shown that rabbit or human antibodies against Y4 toxin can prevent cell death in response to the toxin (P. Baehni, C. C. Tsai, W. McArthur, N. S. Taichman, and R. Genco, Int. Assoc. Dent. Res. **58**, Special Issue A, Abstract 116, 1979). Under these conditions antibodies presumably blocked binding of toxin to the cells. Unfortunately the effects of rabbit and human sera could not be evaluated in the present investigation because both heated (56°C, 30 min) rabbit or human sera in themselves caused significant release of LDH from monocytes. Analogous but still unexplained effects of heated human serum have been observed by others (15).

From the results of experiments with compounds which can affect various monocyte functions, it is apparent that inhibition of protein synthesis (by cycloheximide) or energy metabolism (by sodium fluoride, sodium azide, or 2-

deoxy-D-glucose) did not protect the cells against Y4 toxin. Likewise various anti-inflammatory agents which modulate cytoskeletal integrity, lysosomal integrity, or prostaglandin synthesis and release were without apparent effect on inhibiting cell death. These data again focus attention to the cell membrane as a critical site of attack of the toxin. As yet we have not defined the mechanism of binding or the molecular alterations induced as a consequence of toxin interacting with the membrane. Furthermore, the fate of the toxin during the course of its association with monocytes remains unknown. We are currently purifying the toxin, and once this has been accomplished we should be better able to address these unresolved issues.

PMN and macrophage exudation is one characteristic feature of periodontal disease (27). These cells may serve in the defense of the host against microbial insult in the gingival area. Recent developments suggest that patients with juvenile periodontitis may have impaired PMN chemotaxis which would conceivably increase their susceptibility to periodontal disease (5, 6, 18). Although the role of Y4 in the etiology of juvenile periodontitis is not clear, this organism can be found in relatively high frequency in the subgingival plaques in patients with this disorder compared with other forms of periodontal disease (25, 26, 41; A. C. R. Tanner, R. A. Visconti, and S. S. Socransky, Int. Assoc. Dent. Res. **58**, Special Issue A, Abstr. no. 120, 1979). The toxin which can be extracted from Y4 is capable of destroying isolated human peripheral blood PMNs (45) and gingival crevice PMNs (N. S. Taichman and J. M. A. Wilton, submitted for publication). If comparable events occur in vivo, it would be anticipated that this might lead to defective resistance to dental plaque. Monocytes (macrophages) serve critical roles in antibacterial defense, the immune response, wound healing and remodeling, etc. If Y4 toxin destroys these cells in vivo, this might be accompanied by undesirable local consequences to antimicrobial defense. At the other extreme Y4-poisoned PMNs and monocytes release lysosomal constituents into the extracellular milieu. The indiscriminate release of pro-inflammatory products from these cells can well be postulated as a mechanism of cell and tissue damage in numerous disorders, including periodontal disease.

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