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Cytokine Response by Human Monocytes to *Clostridium difficile* Toxin A and Toxin B

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***Clostridium difficile* toxins A and B isolated from strain VPI 10463 were tested for induction of cytokine release by human monocytes. Toxin B at 10^{-12} M activated human monocytes as measured by release of interleukin-1 (IL-1), tumor necrosis factor (TNF), or IL-6. These effects of toxin B were heat labile (51°C, 30 min). Toxin B was as effective as bacterial lipopolysaccharides in inducing IL-1β but less effective in inducing TNF or IL-6. Toxin B and lipopolysaccharides were synergistic in induction of IL-1β, TNF, and IL-6. The toxin A preparation used was 1,000-fold less active than toxin B. Apart from the difference in activity, the two toxins showed identical patterns of reaction and there was no synergism between them. A short pulse with toxin B was sufficient to trigger IL-1 release. Toxin B was also extremely toxic for monocytes. The toxicity and the induced proinflammatory monokines (IL-1 and TNF) may contribute to the pathogenic mechanisms of *C. difficile* infection and pseudomembranous colitis.**

Pseudomembranous colitis is an acute, often antibiotic-associated, inflammatory bowel disease due to opportunistic growth of *Clostridium difficile* (3, 21). Serious clinical symptoms can include protein-losing enteropathy, severe fluid loss, and toxic megalocolon (32). Toxic products of *C. difficile* have been shown to have cytopathic effects (28) and cause colitis (3, 29), edema, respiratory arrest, and death upon in vivo administration (47). They have long been known to be heat labile (29, 47). Although the bacteria are noninvasive, their toxins seem to enter the mucosal barrier since they can induce an antibody response by the host (53). Two exotoxins have been described (49). The genes for *C. difficile* toxins A and B were cloned recently (2, 16). The gene for toxin A was reported to encode 2,710 amino acids (molecular mass, 308 kDa), and that for toxin B was reported to encode 2,366 amino acids (molecular mass, 269 kDa).

The pathophysiological mechanism of *C. difficile* colitis is not completely understood. Various cytopathic and cytotoxic effects have been described. Clostridial toxins were reported to induce morphological changes in mammalian cell lines (13–15, 17, 55) and affect metabolic functions (45), as well as tight-junction permeability (22), of cell lines. The effects of toxins A and B could be differentiated by these criteria (15, 45). Toxin B was roughly 1,000-fold more active than toxin A (4). The in vitro effects of both toxins were found to be heat labile (48).

Less is known about the influence of clostridial toxins on the immune system. Mitogen- and antigen-driven proliferation of freshly prepared human T cells was inhibited, whereas interleukin-2 (IL-2)-driven T cell proliferation remained unaffected (13), indicating that the effects of the toxins on human T cells were probably mediated by human monocytes. It has also been reported (34) that toxin A at 10^{-9} M could induce IL-1 in elicited peritoneal mouse macrophages. This cytokine was assumed to mediate the stimulatory effect on murine B cells attributed to toxin A. Thus, an understanding of clostridial toxicity would include proinflammatory cellular products (like monokines), in addition to the known direct cytotoxicity to various cell types of the intestinal epithelium.

Several different bacterial toxins have also been shown to exert both cytocidal and immunomodulatory effects on monocytes-macrophages, like mediator induction. Among these toxins are *Pseudomonas aeruginosa* exotoxin A (35, 40, 44), *Escherichia coli* hemolysin (9), and staphylococcal alpha-toxin (8, 33). Induction of IL-1 and tumor necrosis factor (TNF) was shown for *Staphylococcus aureus* toxic shock syndrome toxin 1 (23, 38, 39). Synergism of toxic shock syndrome toxin 1 with lipopolysaccharide (LPS) was also shown (5). No cytotoxicity of toxic shock syndrome toxin 1 to monocytes was reported. Other clostridial toxins, like *C. botulinum* toxin type D, inhibited release of TNF by macrophages after LPS activation (24) or, like *C. perfringens* delta toxin, killed macrophages (25).

The small amounts of bacterial toxins required for immunomodulatory functions suggest specific high-affinity binding to a receptor. Such a mechanism has been established for the activation of certain T cell populations by staphylococcal toxin (19, 26, 43, 46). It has not unequivocally been shown for activation of monocytes-macrophages by either gram-negative or gram-positive bacterial products.

The aim of this study was to determine the effects of clostridial toxins A and B on human monocytes and their cytokines IL-1α, IL-1β, TNF, and IL-6. These monokines are known to exert potent proinflammatory and cytotoxic effects (37), mediate septic shock (7), induce the acute-phase reaction (1), and initiate coagulation (52). They may be the mediators of proinflammatory and cytotoxic effects in the pathophysiological mechanisms of *C. difficile* disease or act synergistically with direct effects of clostridial toxins.

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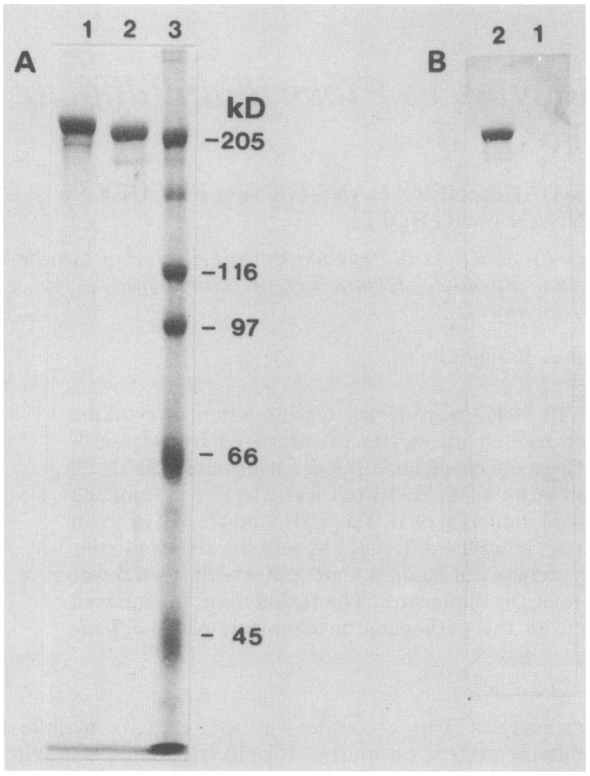

FIG. 1. Purity of *C. difficile* toxin A and B preparations (SDS-polyacrylamide gel electrophoresis and immunoblotting). Panels: A, SDS-polyacrylamide gel electrophoresis; B, Western immunoblot of a gel run in parallel to that shown in panel A. A murine antiserum monospecific for *C. difficile* toxin B was used. Lanes: 1, *C. difficile* toxin A (5 μg); 2, *C. difficile* toxin B (5 μg); 3, molecular mass standards (in kilodaltons).

MATERIALS AND METHODS

***Clostridium difficile* toxins A and B.** Purification of toxins was done by the method described previously (13, 54), with some modifications. *C. difficile* VPI 10463 was grown in brain heart infusion (Difco, Detroit, Mich.) for 72 h at 37°C in a 3-liter volume. The culture supernatant was clarified by centrifugation at 10,000 × *g* for 15 min at 4°C. Toxins A and B were precipitated successively by addition of solid $(NH_4)_2SO_4$ to achieve either 40 or 70% saturation. Each precipitate was collected by centrifugation at 10,000 × *g* for 30 min. The protein pellets were dissolved in 50 ml of 50 mM Tris-HCl (pH 7.5), dialyzed against 50 mM Tris-HCl–25 mM NaCl (pH 7.5) overnight at 4°C, and further purified by Mono Q anion-exchange chromatography (fast protein liquid chromatography system; Pharmacia, Freiburg, Germany). With a linear salt gradient in 50 mM Tris-HCl (pH 7.5), toxins A and B eluted at 180 and 550 mM NaCl, respectively. Fractions were checked with a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel (27) stained with Coomassie brillant blue and by cytotoxicity assay (13, 54). The purity of toxin A and B preparations is shown in Fig. 1A. The high-molecular-weight standard was from Sigma (Munich, Germany). Immunoblotting (51) was done with a murine antiserum raised in our laboratory against SDS-polyacrylamide gel electrophoresis-purified *C. difficile* toxin B. Immunostaining was performed with alkaline phosphatase-labeled anti-mouse immunoglobulin G (Dianova, Hamburg, Germany) and 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium chloride (Boehringer GmbH, Mannheim, Germany) as substrates. No contaminating *C. difficile* toxin B was detected in the toxin A preparation when it was tested by immunoblotting (Fig. 1B).

Stock solutions of toxins A (150 μg/ml) and of B (50 μg/ml) were kept aliquoted at −80°C for up to 6 weeks. Prolonged storage (6 months) at −80°C resulted in loss of >90% of the biological activity of toxin B. In the (undiluted) stock toxin preparations, endotoxin could be detected after 10 min of treatment at 75°C in amounts of less than 1.8 ng/ml as measured by a modified chromogenic *Limulus* amebocyte lysate assay (kindly done by D. Berger, Ulm, Germany) as described in reference 6. The lysate was from Pyroquant, Walldorf, Germany, and the chromogen (Pefachrom) was from Pentapharm, Basel, Switzerland. The detection limit was 1.5 pg of *E. coli* O55:B5 LPS per ml. Protein was determined by the method of Bradford (11) with bovine serum albumin (Behring, Marburg, Germany) as the standard. Nonfiltered toxin A and B preparations proved to be free of bacterial contamination; they were, however, used after filtration (0.22-μm pore size; Millex-GV; Millipore, Molsheim, France) and dilution in culture medium supplemented with LPS-free (36) fetal calf serum (lot 028011; Flow, Meckenheim, Germany). Polyclonal goat anti-*C. difficile* toxin serum (lot 304) was from Paesel+Lorei, Frankfurt am Main, Germany.

Human monocytes and monokine production assay. Monocytes were separated from buffy coat layers of CPDA-1 (citric acid, sodium citrate, sodium dihydrogen phosphate, glucose, adenine hydrochloride) anticoagulant-treated blood by density gradient centrifugation and plastic adherence under LPS-free conditions as described previously (18). The cell preparations were monitored by fluorescence-activated cell sorter (EPICS; Coulter, Krefeld, Germany) analysis; purity of monocytes was greater 80% ($CD14^+$); contaminating cells were T (<10% $CD3^+$), B (<15% $CD20^+$), and natural killer (<5% $CD58^+$) cells. Monocyte preparations which did not meet these criteria were not used, except for monocyte toxicity experiments, for which mononuclear cell preparations containing 60% monocytes were used for technical reasons. Monokine production was done in 96-well microculture plates (Falcon 3075; Becton Dickinson, Heidelberg, Germany) using a 200-μl monocyte suspension (3×10^5 to 5×10^5 cells per ml) per well. The monocytes were cultured overnight at 37°C in 5% CO_2 and 98% humidity with clostridial toxins or LPS from *Salmonella typhi* O901 (Difco), as indicated in Results. Culture medium was RPMI 1640 (074-01800; Life Technologies, Eggenstein, Germany) supplemented as described in reference 18 and further supplemented by 24 mM $NaHCO_3$. Cultures were supplemented by 10% fetal calf serum. Testing was done in duplicate. Supernatants were aliquoted and stored at −20°C until being tested for monokines.

Monokine assays. IL-1 was measured by an enzyme-linked immunosorbent assay (ELISA) from Endogen, Boston, Mass., for IL-1α, an ELISA from Biochrom, Berlin, Germany, for IL-1β, the thymocyte costimulator assay (18), or a combined assay (20) using EL4 and CTLL cell lines (EL4-CTLL assay). IL-6 was measured by ELISA (Quantikine; R&D Systems, Minneapolis, Minn.) or hybridoma growth induction using the 7TD1 cell line (18), as indicated in Results. For the biological monokine assays, units of IL activity were determined by computerized logit transformation and comparison to the international standards for IL-1β and IL-6 (National Institute for Biological Standards and Control, Potters Bar, England). For the combined EL4-

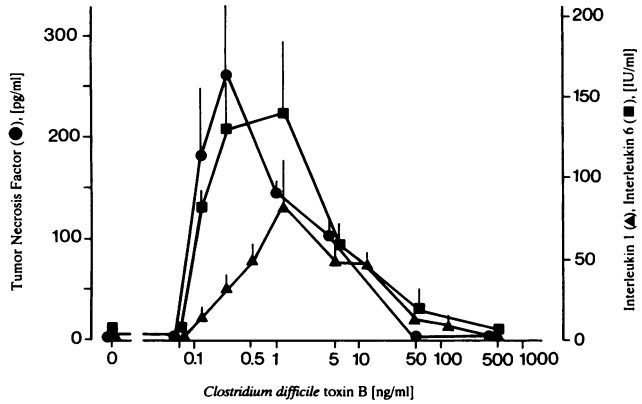

FIG. 2. Dose response of monokine release by human monocytes induced by *C. difficile* toxin B. Different doses of toxin B (abscissa) were incubated with human monocytes. In the culture supernatants, the activities of IL-1 (measured by thymocyte assay), TNF (measured by ELISA), and IL-6 (measured by 7TD1 hybridoma growth induction) were determined. Amounts of cytokines induced are given on the ordinate (mean + standard deviation; n = 2).

CTLL assay, EL4 and CTLL cells were expanded, aliquoted, stored in liquid nitrogen (10% dimethyl sulfoxide [Merck AG, Darmstadt, Germany], 10% fetal calf serum, 80% RPMI 1640), and used for the cytokine assays immediately after thawing. Supernatants to be tested were titrated, EL4 cells were added to a final concentration of 6×10^5/ml in a total volume of 150 μl of RPMI 1640 containing 5% fetal calf serum, and the mixture was incubated for 24 h at 37°C. Then the culture was frozen and thawed, 50 μl of a CTLL suspension (final concentration, 10^5/ml) was added, and the mixture was incubated for 48 h at 37°C. The culture was pulsed with [^{3}H]thymidine for the final 24 h. Biological cytokine assays were done in duplicate and titrated and tested in at least four different dilution steps. Testing for TNF was done by ELISA (T Cell Sciences, Cambridge, Mass.). Preparations of toxins A and B were negative in all of the cytokine assays used. Therefore, neither toxin affected either IL-1- or IL-2-driven proliferation of thymocytes, IL-2 secretion by EL4 cells, or IL-2-driven proliferation of CTLL cells.

Testing of cell viability. Monocyte viability was tested by either incorporation of acridine orange (0.4 mg/ml) and ethidium bromide (0.5 mg/ml) (Sigma; inverted fluorescence microscope, Zeiss, Oberkochen, Germany), the trypan blue dye exclusion method, or fluorescence-activated cell sorter analysis using anti-CD14 (My4; Coulter) antibodies.

RESULTS

The purity of the toxin A and B preparations from the gram-positive bacillus *C. difficile* is shown in Fig. 1. These preparations were tested for the cytokine response induced in human monocytes.

Monokine release by human monocytes: (i) Effect of *C. difficile* toxin B and synergistic effects of toxin B and LPS. Human monocytes were cultured with various concentrations of toxin B, and the monokines induced were measured (Fig. 2). Toxin B induced cytokines as measured by thymocyte assay at a concentration of 0.5 ng/ml, which corresponded on a molar basis to an effective dose of 10^{-12} M. TNF (ELISA) and IL-6 (7TD1 hybridoma growth induction)

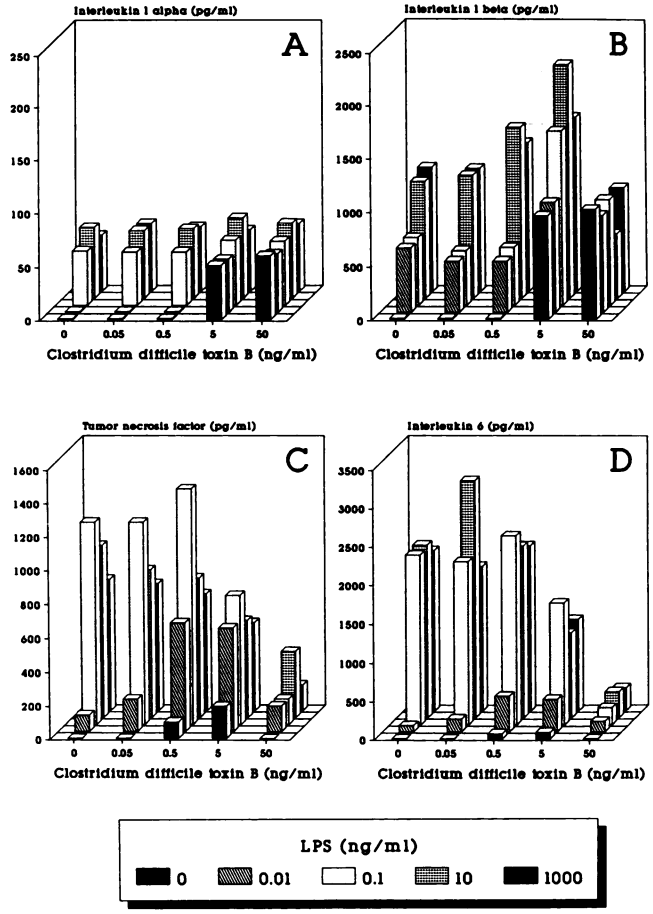

FIG. 3. Dose response of monokine release by human monocytes induced by *C. difficile* toxin B and checkerboard titration for synergistic effects of *C. difficile* toxin B and *S. typhi* LPS. Various concentrations of toxin B (x axes) and LPS (z axes) were added to monocytes, which were cultured in the presence of the toxins for 24 h at 37°C. Identical monocyte supernatants were tested for IL-1α, IL-1β, TNF, and IL-6 by ELISAs. Amounts of monokines released are depicted on the y axes. (A) IL-1α. (B) IL-1β. (C) TNF. (D) IL-6. The results from one of two experiments are shown.

were induced by similar concentrations of toxin B. Concentrations of >5 ng of toxin B per ml were inhibitory. Next, the synergism of toxin B with LPS was tested by checkerboard titration (Fig. 3). Both LPS and toxin B induced release of IL-1α (up to 50 pg/ml; Fig. 3A) and IL-1β (up to 1,200 pg/ml; Fig. 3B) in comparable amounts. Synergism was observed only for induction of IL-1β. Synergism was strongest when both agents were present in optimal concentrations. A pattern similar to that of IL-1β release (as measured by ELISA) was found when the biological activity of IL-1 was tested by EL4-CTLL assay (data not shown), reflecting the dominant role of IL-1β. Toxin B by itself also induced TNF and IL-6 (Fig. 3C and D). The amounts were, however, far lower than the amounts induced by optimal concentrations of LPS. Toxin B (0.5 to 5 ng/ml) was synergistic with suboptimal concentrations of LPS for induction of TNF and IL-6.

(ii) Time kinetics of IL-1β release. Figure 4 shows the kinetics of IL-1β release by toxin B-exposed human monocytes. Cytokine release in response to 5 ng of toxin B did not occur before 3 h after exposure; it was, however, about

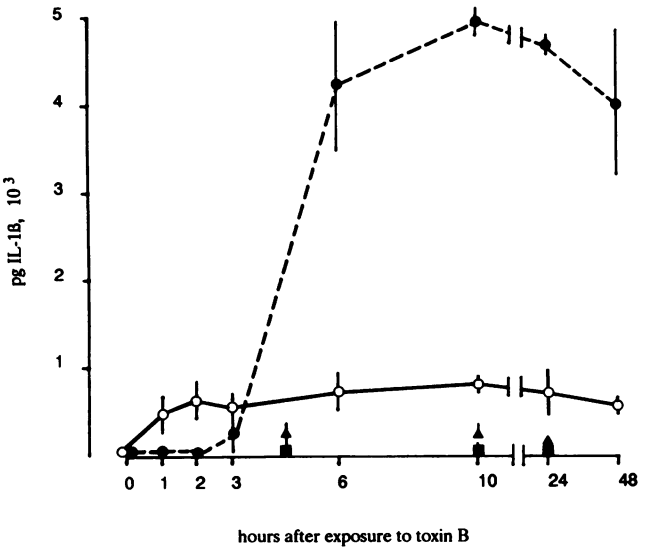

FIG. 4. Time kinetics of IL-1β release of human monocytes induced by *C. difficile* toxin B. Monocytes were incubated with toxin B for various periods of time (abscissa), and IL-1β was determined in supernatants by ELISA (ordinate, mean ± standard deviation; $n = 2$). Symbols: ○, toxin B at 500 ng/ml; ●, toxin B at 5 ng/ml; ▲, toxin B at 5 ng/ml and cycloheximide at 10 μg/ml; ■, toxin B at 5 ng/ml and actinomycin D at 10 μg/ml.

complete after 6 h. In contrast, 500 ng of toxin B caused rapid release of low amounts of IL-1β, with no further release after 2 h. The rapid cessation of IL-1β release can be explained by cytotoxicity (see below). Very brief contact with toxin B was sufficient to trigger monocytes: cells which were pulsed with 500 ng of toxin B and immediately washed still died within 24 h but produced large amounts of IL-1β, comparable to those induced by 5 ng of toxin B per ml (data not shown). Inhibitors of protein synthesis (cycloheximide and actinomycin D) prevented cytokine formation and release by monocytes, indicating that protein synthesis is required for induction of monokines by toxin B.

(iii) Effect of *C. difficile* toxin A. The toxin A preparation was also able to induce IL-1, TNF, and IL-6 in human monocytes (Table 1). The concentrations required were, however, about 1,000-fold higher for toxin A (≥150 ng/ml or $\geq 10^{-9}$ M) than for toxin B. Toxin A showed no synergism with toxin B. Small amounts of toxin A, which by themselves did not induce mediator release, showed synergism with suboptimal doses of LPS.

TABLE 2. Effect of heat-treated *C. difficile* toxin B on cytokine release by human monocytes

Concn (ng/ml) of:		IL-1 release[a] (IU/ml; mean ± SD, $n = 2$)			
Toxin B	LPS	4°C	37°C	46°C	51°C
50	0	80 ± 6	110 ± 21	213 ± 46	0 ± 0
5	0	206 ± 32	201 ± 54	0 ± 0	0 ± 0
0	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
50	0.1	85 ± 7	89 ± 7	269 ± 83	43 ± 16
5	0.1	329 ± 13	263 ± 18	198 ± 25	42 ± 1
0	0.1	54 ± 10	44 ± 15	28 ± 6	24 ± 2

[a] IL-1 was measured by the thymocyte assay. Comparable results were obtained when TNF and IL-6 were tested by ELISA (data not shown).

(iv) Toxin inhibition by antiserum. When anti-*C. difficile* toxin serum (final dilution, 1:1,000) was incubated with toxin B (50, 5, and 0.5 ng/ml) or A (300 ng/ml) for 2 h at 37°C, all of the toxin doses tested were unable to induce IL-1. The synergistic effect of toxins with exogenously added LPS was also abrogated by antiserum. As a control, IL-1 induction by exogenous LPS (final concentration, 1 ng/ml) was not affected by anti-*C. difficile* toxin serum (data not shown).

Heat lability of *C. difficile* toxins. Heat lability of clostridial toxins has repeatedly been reported. To test the effect of heat treatment on the ability of toxin B to induce cytokines, aliquots of toxin B were incubated in water baths at various temperatures (4 to 51°C) and then assayed for activation of monocytes. Exposure to a temperature of 51°C completely abolished the cytokine-inducing effect of toxin B (Table 2). The same pattern of heat lability of toxin B was also found when it was tested for synergistic effects with LPS. Comparable results were found for TNF and IL-6 (tested by ELISA; data not shown). Toxin A was tested as described for toxin B. Release of monokines (IL-1, TNF, and IL-6) was effectively prevented by exposing toxin A to a temperature of 51°C (Table 3). The results obtained with heat-treated toxins can also be interpreted as controls demonstrating that the effects attributed to toxins A and B were not caused by inadvertent contamination by LPS, since LPS is known to be inordinately resistant to heat.

Cytotoxicity of toxin B to human monocytes. The cytotoxic effects of toxin B on human monocytes were tested by fluorescence-activated cell sorter analysis by using a CD14 monoclonal antibody (My4; Coulter). Monocytes were ex-

TABLE 1. Effect of *C. difficile* toxin A on cytokine release by human monocytes and synergism with *C. difficile* toxin B and *S. typhi* LPS

Toxin A concn (ng/ml)	Cytokine release[a] (mean ± SD; $n = 2$)								
	Toxin A			Toxins A and B (5 ng/ml)			Toxin A and LPS (0.1 ng/ml)		
	IL-1 (IU/ml)	TNF (pg/ml)	IL-6 (pg/ml)	IL-1 (IU/ml)	TNF (pg/ml)	IL-6 (pg/ml)	IL-1 (IU/ml)	TNF (pg/ml)	IL-6 (pg/ml)
1,500	233 ± 24	698 ± 280	207 ± 83	341 ± 181	363 ± 53	79 ± 23	644 ± 284	3,375 ± 177	2,170 ± 1,287
150	21 ± 30	0 ± 0	57 ± 23	233 ± 163	313 ± 53	91 ± 58	297 ± 158	2,313 ± 88	2,900 ± 141
15	0 ± 0	0 ± 0	25 ± 35	174 ± 91	263 ± 71	89 ± 62	97 ± 11	1,532 ± 309	3,160 ± 85
1.5	0 ± 0	0 ± 0	0 ± 0	160 ± 82	228 ± 4	77 ± 50	77 ± 33	1,338 ± 124	2,900 ± 141
0	0 ± 0	0 ± 0	0 ± 0	228 ± 162	257 ± 62	63 ± 25	111 ± 66	1,625 ± 354	2,530 ± 806

[a] IL-1 was determined by thymocyte assay, and TNF and IL-6 were determined by ELISA.

TABLE 3. Effect of heat-treated *C. difficile* toxin A on cytokine release by human monocytes[a]

Temp (°C)	Mean concn ± SD of:		
	IL-1 (IU/ml)	TNF (pg/ml)	IL-6 (pg/ml)
4	182 ± 31	ND[b]	ND
37	184 ± 42	1,362 ± 442	480 ± 382
46	84 ± 48	838 ± 654	255 ± 262
51	0 ± 0	0 ± 0	12 ± 17

[a] Toxin A was used at 1,500 ng/ml (n = 2). IL-1 was determined by thymocyte assay, and TNF and IL-6 were determined by ELISA. In the absence of toxin A and at 56°C, no cytokines were detected.
[b] ND, not determined.

TABLE 4. Toxicity of *C. difficile* toxin B for human monocytes

Toxin B concn (ng/ml)	% CD14-positive cells[a] (mean ± SD)		
	Before exposure (n = 4)	After 5 h of exposure (n = 4)	After 24 h of exposure (n = 2)
0	53 ± 1	43 ± 2	47 ± 2
5	ND[b]	10 ± 5	1 ± 2
50	ND	1 ± 1	0 ± 0
500	ND	0 ± 1	1 ± 1

[a] Tested by fluorescens activated cell sorter (EPICS; Coulter) using My4 monoclonal antibody.
[b] ND, not determined.

posed to different doses of toxin B, and the remaining cells were analyzed 5 and 24 h later. For technical reasons, mononuclear preparations containing about 60% monocytes were used in these experiments. Table 4 shows that after 5 h in the presence of 500 ng of toxin B per ml, virtually no $CD14^+$ cells were left. Even with a dose of 5 ng/ml, no $CD14^+$ cells were detectable after 24 h and three-fourths of the monocytes had died within 5 h. It should be noted that monocytes treated with 500 ng of toxin B per ml released small amounts of IL-1β (<1,000 pg/ml) before they died (data not shown). This corresponds to the data shown in Fig. 2. The remaining cells (apparently all lymphocytes by morphology and by acridine orange staining) were not able to produce any of the monokines under test when they were washed and recultivated after 5 h of treatment with 500 ng of toxin B per ml. Furthermore, not a trace of monokines was released when the remaining cells were washed, recultivated for 24 h, and then restimulated with LPS or toxin B for a further 24 h (data not shown). Additional studies using highly enriched monocyte populations and acridine orange staining or trypan blue exlusion confirmed that the elimination of monocytes by toxin B was total (data not shown).

Effect of human serum on toxin B-induced monocyte activation. LPSs, the major toxins of gram-negative bacteria, are thought to mediate their toxic effects by monocyte activation and release of monokines (30). Recently (12, 18), it was shown that serum could inhibit this effect of monocyte activation, indicating a mechanism of detoxifying LPS which involved human lipoproteins. Lipoproteins can inactivate LPS, probably because of hydrophobic interactions (31). Human low-density lipoproteins were shown to bind to *S. aureus* alpha-toxin and partially inactivate this toxin in regard to its hemolytic activity (10). An assay (18) for toxin inhibition by human serum lipoproteins was applied to *C. difficile* toxin B (Table 5). Toxin B was either incubated with serum for 24 h and then added to monocytes or added to the cultures together with the serum. Both sets were then incubated for 24 h, and the cytokines induced were measured in the supernatants. The activity of toxin B incubated for 24 h at 37°C with serum was only marginally reduced, probably because of the heat lability of toxin B, showing also that toxin B is quite resistant to exposure to a temperature of 37°C for 24 h. A comparable reduction of activity was seen when toxin B was incubated with lipoprotein-free serum, showing that there was no inactivation of toxin B by lipoproteins. As a control, inactivation of LPS by human serum and lack of inactivation of LPS by lipoprotein-free serum are shown.

DISCUSSION

A recent study (34) reported that clostridial toxin A could stimulate mouse macrophages to release IL-1. In the present investigation, we analyzed the effect of clostridial toxins A and B on human monocytes. We found that clostridial toxins are potent inducers of monokines (IL-1, TNF, and IL-6). Clostridial toxin B is also a very potent toxin for human monocytes. Induction of monokines requires a minimal effective dose of 10^{-12} M toxin B. Thus, it is similar in effectiveness to LPS from gram-negative bacteria on a molar basis. There is also a synergism of clostridial toxin B and LPSs.

Massive induction of monokines and toxicity for monocytes may both take part in the pathogenic mechanism of *C. difficile* infections. Local release of monokines (e.g., TNF) in the intestinal mucosae is known to cause cell alterations, activate endothelial cells, and increase inflammation (41, 42, 50). The direct cytopathological effects of toxin B for a variety of cells (17) might thus be amplified by massive release of monokines. In this respect, the demonstrated synergism of *C. difficile* exotoxins with endotoxins (LPS) from gram-negative bacteria may be important. Generally, endotoxins can be assumed to be present simultaneously when clostridial toxins occur in the intestines. A combination of both toxins might then determine the pathological sequelae, explaining why the amounts of clostridial toxins that occur may be insufficient to predict the severity of *C. difficile* disease. Recruitment of proinflammatory monokines could be restricted to the intestinal wall; in severe cases, however, it may systemically mediate the acute-phase reaction (1), induce coagulation (52) and contribute to shock (7).

Monocytes proved to be very sensitive to the toxic effects of toxin B. Hence, in vivo toxin B may first cause monocytes to secrete proinflammatory products and then proceed to kill the cells, rendering them unable to fulfill other important functions, like phagocytosis and extracellular killing of bacteria. In other words, a large fraction of the monocytes entering the scene may foster inflammation but be hampered from fighting infection.

Induction of monokines in association with direct killing of monocytes may represent a common pathway of toxicity for a variety of bacterial toxins. *C. difficile* toxin B shares these features with *P. aeruginosa* toxin A (35), *E. coli* hemolysin (9), and staphylococcal alpha-toxin (8). Induction of monokines and synergism with LPS in the absence of cytocidal effects for monocytes were shown (38) for *S. aureus* toxic shock syndrome toxin 1.

We also pondered the possibility that toxin B can be a useful tool for removal of monocytes from mixed cell populations or suppression of endogenous production of mono-

TABLE 5. Lack of inactivation of *C. difficile* toxin B by human serum lipoproteins

Toxin	Dose (ng/ml)	IL-1 release[a] (IU/ml; mean ± SD, $n = 2$)			
		Human serum		Lipoprotein-free serum	
		Toxin incubated with serum[b]	Toxin added after monocytes	Toxin incubated with lipoprotein-free serum[b]	Toxin added after monocytes
Toxin B	50	67 ± 1	72 ± 22	53 ± 13	74 ± 1
	5	235 ± 29	285 ± 4	170 ± 11	226 ± 4
	0.5	9 ± 9	60 ± 18	2 ± 2	27 ± 9
	0.05	0 ± 0	0 ± 0	0 ± 0	2 ± 2
LPS	10	140 ± 14	162 ± 2	158 ± 11	151 ± 16
	1	0 ± 0	139 ± 12	129 ± 27	136 ± 15
	0.1	0 ± 0	70 ± 14	28 ± 11	27 ± 16

[a] IL-1 was measured by thymocyte assay. In the absence of toxin B or LPS, there was no IL-1 release.
[b] Toxin B or LPS was incubated with serum or lipoprotein-free serum for 24 h at 37°C, and then monocytes were added and cultured for 24 h.

kines in mixed cell cultures containing monocytes and lymphocytes. As shown in reference 13, IL-2-dependent growth of human T cells is not affected by toxin B. Indeed, treatment of mixed cultures with toxin B can reliably eliminate all monocytes within hours. Since lymphocytes seem to be utterly resistant to toxin B, this may constitute a suitable way to prepare monocyte-depleted cell populations. It should, however, be noted that an initial wave of IL-1β release induced by toxin B itself cannot be avoided, even when doses of ≥500 ng/ml are used and that toxin B is relatively unstable, decaying, even at −80°C, within a few months.

The monokine induction assay and monocyte toxicity determination by fluorescence-activated cell sorter can both be exploited for detection and quantitation of toxin B. An assay with monocytes would allow testing of large quantities of test samples, e.g., from *C. difficile* culture supernatants. It may be more precise than current assays and does not depend on visual reading of test results, like morphological changes of cells. Specificity could be achieved by use of proper antibodies. The exquisite sensitivity of toxin B to heat would further allow its dissociation from contaminating LPS.

In this study, it was not possible to evaluate with certainty to what extent *C. difficile* toxin A shares the effects ascribed to toxin B. The toxin A preparation used was roughly 1,000-fold less active than the toxin B preparation. This is in accordance with reports in the literature. Apart from the difference in activity, the biological properties of toxin A tested (heat lability and synergism with LPS) were identical to those of toxin B. There was no synergism of toxin A with toxin B. Since neither monospecific inhibitory antibodies nor cloned material was available when this study was done, we are unable to exclude the possibility that the activity found in the toxin A preparation was due to minor contamination with toxin B. In any case, the biological activity of toxin A at the cellular level of human monocytes is either far less than that of toxin B or completely absent.

The mechanism of monocyte activation by *C. difficile* exotoxin is unknown. The extremely low concentration of toxin B required for monocyte activation suggests a highly selective mechanism, in contrast to that of mitogenic lectins, which bind to many structures on monocytes and require >1,000-times-larger concentrations. Monocyte activation by toxin B seemed to be mediated by nonhydrophobic moieties, since toxin B could not be inactivated by lipoproteins and was found to be synergistic with LPS.

In conclusion, we showed that *C. difficile* toxin B is a potent activator of human monocytes. Toxin B is also cytocidal to human monocytes. Toxin B shares the combination of both features with some other bacterial toxins. Monokines are known to increase intestinal inflammation and participate in shock. Their induction may thus contribute to the occurrence of local and systemic damage in enteropathy because of clostridial toxins. The exquisite sensitivity of monocytes, along with the total insensitivity of lymphocytes, towards the toxic effects of toxin B may also make toxin B a useful scientific tool.

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