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Comparative Study of Cytokine Release by Human Peripheral Blood Mononuclear Cells Stimulated with *Streptococcus pyogenes* Superantigenic Erythrogenic Toxins, Heat-Killed Streptococci, and Lipopolysaccharide

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The differences between toxic or septic shocks in humans during infections by streptococci and gram-negative bacteria remain to be fully characterized. For this purpose, a quantitative study of the cytokine-inducing capacity of *Streptococcus pyogenes* erythrogenic (pyrogenic) exotoxins (ETs) A and C, heat-killed *S. pyogenes* bacteria, and *Neisseria meningitidis* endotoxin (lipopolysaccharide [LPS]) on human peripheral blood mononuclear cells (PBMC) and monocytes has been undertaken. The levels of interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), and TNF-β induced by these bacterial products and bacteria were determined by using cell supernatants. The capacity of ETs to elicit the monocyte-derived cytokines IL-1α, IL-1β, IL-6, and TNF-α was found to depend on the presence of T lymphocytes, because of the failure of purified monocytes to produce significant amounts of these cytokines in response to ETs. PBMC elicited large amounts of these cytokines, as well as IL-8 and TNF-β, with an optimal release after 48 to 96 h. The most abundant cytokine produced in response to ETA was IL-8. In contrast to the superantigens ETA and ETC, LPS and heat-killed streptococci stimulated the production of significant amounts of IL-1α, IL-1β, IL-6, and TNF-α, with optimal production after 24 to 48 h in monocytes, indicating no significant involvement of T cells in the process. ETs, but neither LPS nor streptococci, were potent inducers of TNF-β in PBMC. This study outlines the differences in the pathophysiological features of shock evoked by endotoxins and superantigens during infection by gram-negative bacteria and group A streptococci, respectively. The production of TNF-α was a common pathway for LPS, streptococcal cells, and ETs, although cell requirements and kinetics of cytokine release were different.

Despite extensive investigations over the past 20 years on the pathophysiological effects of *Streptococcus pyogenes* erythrogenic toxins, also called pyrogenic exotoxins or scarlet fever toxins (2, 4, 6, 12, 24, 36, 39), the cellular and molecular mechanisms of action of these proteins are still not completely understood (4, 6, 22). Several lines of evidence suggest that erythrogenic toxins (ETs), which are classically considered to comprise three antigenic forms, A, B, C, are, at least for the A and C serotypes, the major molecular effectors involved in the pathogenesis of scarlet fever and streptococcal toxic shock syndrome (STSS) (2, 4, 11, 21, 22, 27, 35, 39, 41). Since the first reports on this syndrome in the past decade (39, 41), a large number of cases of this severe disease have been recorded (4, 25, 38). STSS, which is similar to fulminant/septic scarlet fever (4, 25) described in the first half of this century, has much in common at the clinical and pathophysiological levels with staphylococcal toxic shock syndrome (TSS) elicited by *Staphylococcus aureus* toxic shock syndrome toxin 1 (TSST-1) or enterotoxins or both (2, 12, 26, 34). However, the two syndromes differ by their epidemiological backgrounds and mortality rates, which are much higher in STSS (ca. 30%) (4) than in TSS (ca. 6%) (2).

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Streptococcal ETs and staphylococcal enterotoxins exhibit significant structural and antigenic homology (2, 26). They also share, as well as TSST-1, a number of major biological properties such as pyrogenicity, immunosuppressive effects, and enhancement of the susceptibility of experimental animals to lethal shock by endotoxins. These toxins also bind in their native state to major histocompatibility complex class II molecules on antigen-presenting cells and elicit in parallel polyclonal expansion of human, murine, and other animal T lymphocytes upon interaction with particular segments of the Vβ elements in the T-cell receptor (2, 6, 22, 26, 29). In this respect, these toxins belong to the so-called superantigen family (2, 26).

Extensive studies on *S. aureus* enterotoxins and TSST-1 clearly showed that by virtue of their in vitro or in vivo interaction with T cells and monocytes/macrophages, these toxins induce massive release of various cytokines, which appear to be the major mediators of TSS (2, 12, 13, 17, 26, 34, 37). Similarly, the septic shock provoked by gram-negative bacteria is also due mainly to the release by immune system cells of various endogeneous mediators including inflammatory factors and a number of cytokines, particularly tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), in response to lipopolysaccharides (LPS) located on the outer membrane of these bacteria (5, 9). In contrast, limited information has been provided so far concerning the capacity of

ETs to induce cytokine production by human immune system cells. This led us to undertake a comparative quantitative study of the in vitro capacity of native and recombinant ETA and ETC, heat-killed group A streptococci, and *Neisseria meningitidis* LPS to induce cytokine production by human monocytes and peripheral blood mononuclear cells (PBMC). The release of TNF-α, TNF-β, IL-1 α, IL-1β, IL-6, and IL-8 was determined as a function of incubation time and of the concentration of ETs, LPS, and a given number of streptococcal cells. We also demonstrate the contribution of T lymphocytes in the in vitro production of monocyte/macrophage-derived cytokines in response to ETs, as previously shown in mice (30).

MATERIALS AND METHODS

Cytokine inducers. Three different cytokine inducers have been investigated: ETA and ETC, *N. meningitidis* LPS, and heat-killed *S. pyogenes* cells. In certain experiments, *S. aureus* TSST-1 (Toxicon, Madison, Wis.) was also used for comparative purposes.

Two ETA preparations (M_r 25,805) purified to apparent homogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were used without discrimination. One preparation was purified from *S. pyogenes* NY-5 culture supernatant (15), and the other was recombinant ETA purified from culture filtrate of *S. sanguis* Challis containing the *speA* gene (16). The two ETA preparations were comparable in their molecular weight, mitogenic activity and cytokine-inducing capacities. These preparations did not contain exogeneous LPS contaminants greater than 340 pg/mg as assessed by the *Limulus* colorimetric reagent (Whittaker, Walkersville, Md.). ETC was purified from culture filtrate of *S. pyogenes* NY-5 as described previously (33). Unless otherwise stated, the monocytes and PBMC were challenged with increasing concentrations of ETA and ETC ranging from 0.2 to 20 μg/ml of cell suspension.

N. meningitidis LPS was kindly provided by Martine Caroff, Paris XI University, Orsay, France. At the LPS concentrations used, the cytokine-inducing capacity of this preparation was not inhibitable by polymyxin B, in contrast to other endotoxins (8, 9). In most experiments, the optimal release of cytokines was obtained at a concentration of 0.2 μg of LPS per ml.

Suspensions of heat-killed cocci of *S. pyogenes* S 84 (Griffith type 3) were prepared by growing the strain overnight at 37°C in Todd-Hewitt broth. The culture was centrifuged at 5,000 × *g* for 10 min, and the cell pellet was washed three times in pyrogen-free saline and suspended in 0.5 ml of this fluid. The turbidity of the suspension was adjusted to an A_{600} of 0.7 in a Beckman U 2000 spectrophotometer. This suspension contained ca. 10^9 CFU/ml and was heat inactivated for 1 h at 70°C.

Washed latex beads (Standard Dow latex, 0.815 μm in diameter; Serva, Heidelberg, Germany) in suspension in RPMI medium were adjusted to the same optical absorbance as that of heat-killed streptococci and used as controls.

Cytokine-producing cells. PBMC were isolated from venous blood of healthy donors (containing 10 IU of heparin per ml), diluted in an equal volume of RPMI 1640 medium, and centrifuged over Ficoll-Hypaque (MSL; Eurobio, Les Ulis, France) for 20 min at 150 × *g* at room temperature. After being washed, PBMC were adjusted to 2.5×10^6 cells per ml in RPMI medium supplemented with antibiotics (100 μg of penicillin per ml and 100 μg of streptomycin per ml), (2 mM) L-glutamine, 2.5% fetal calf serum (Boehringer, Mannheim, Germany), and 5×10^{-5} M 2-mercaptoethanol. The culture medium also contained indomethacin (1 μg/ml) to avoid possible interference with prostaglandin synthesis. The monocytes were separated from PBMC after numeration and nonspecific esterase staining. Then, 5×10^5 nonspecific esterase-positive cells per 0.5 ml of RPMI medium per well were purified by adherence to a plastic surface (24-well multidish plates; Falcon Labware, Oxnard, Calif.). After incubation for 1 h at 37°C, the cells were vigorously washed and adherent cells were further incubated in RPMI medium with 0.2% human serum.

All cytokine-inducing experiments were performed in the presence of 2 μg of polymyxin B per ml to avoid false-positive results from possible LPS contaminations of the reagents used. The stimulators (ETA, ETC, LPS, TSST-1, and heat-killed streptococci) were added at the beginning of the cell culture.

PBMC and monocyte suspensions were incubated in volumes of 500 μl at 37°C in an incubator (7% CO_2–93% air atmosphere) for various periods up to 96 h with the appropriate amounts of stimulators added in volumes of 10 μl. At the end of the incubation periods, cell supernatants were collected, clarified by centrifugation, and stored at −20°C until used for assay of their cytokine content. Triplicate samples were processed for each dose of stimulant used. Each experiment was performed on cells from 3 to 14 donors.

ELISA of IL-1α. IL-1α determination was carried out by a specific enzyme-linked immunosorbent assay (ELISA) method (31) with two anti-recombinant human IL-1α monoclonal antibodies raised in mice: an immunoglobulin G1 (no. 28-9) and an immunoglobulin G2b (no. 2-12), which do not recognize the same epitope on IL-1α. The levels of IL-1α in the samples were calculated by reference to the standard curve. The detection limit of IL-1α was 30 pg/ml.

Radioimmunoassay of IL-1β. A radioimmunoassay procedure similar to that previously described was used (31).

Radioimmunoassay of TNF-α. The radioimmunoassay of TNF-α was based on a competitive inhibition assay for human TNF-α as described previously (31).

ELISA of TNF-α. The ELISA of TNF-α has been developed by using a monoclonal antibody from Bayer-Pharma, Puteaux, France, and a rabbit polyclonal antibody kindly provided by C. Rougeot, Institut Pasteur. This assay was performed as previously described (9).

ELISA of TNF-β. The test kit Quantikine-human TNF-β immunoassay was purchased from R & D System Europe, Abingtdon, United Kingdom, and used as specified by the manufacturer.

Assay of IL-6. The IL-6 assay was determined by using the IL-6-dependent 7TD1 mouse hybridoma which was a gift of D. J. Van Snick, Ludwig Institute, Brussels, Belgium. IL-6 activity was estimated as described previously (31).

ELISA of IL-8. An ELISA specific for IL-8 was set up with a monoclonal antibody (MAb 4), prepared by J-C. Mazié, Hybridolab, Institut Pasteur, Paris, France, against recombinant human IL-8 (Immugenex, Los Angeles, Calif.) and a rabbit polyclonal anti-IL-8 antiserum kindly provided by N. Vita, Sanofi Elf Biorecherche, Labège, France. The assay was performed as previously described (27).

RESULTS

Cytokine induction by ETA and LPS. PBMC and monocytes were challenged as described in Materials and Methods with 0.2, 2, and 20 μg of ETA (native or recombinant) per ml or with 0.2 μg of *N. meningitidis* LPS per ml. The assay of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and TNF-β in the supernatants of unstimulated cells or in those of ETA- or LPS-stimulated cells was performed after various times of incubation (up to 96 h in most experiments) as indicated below.

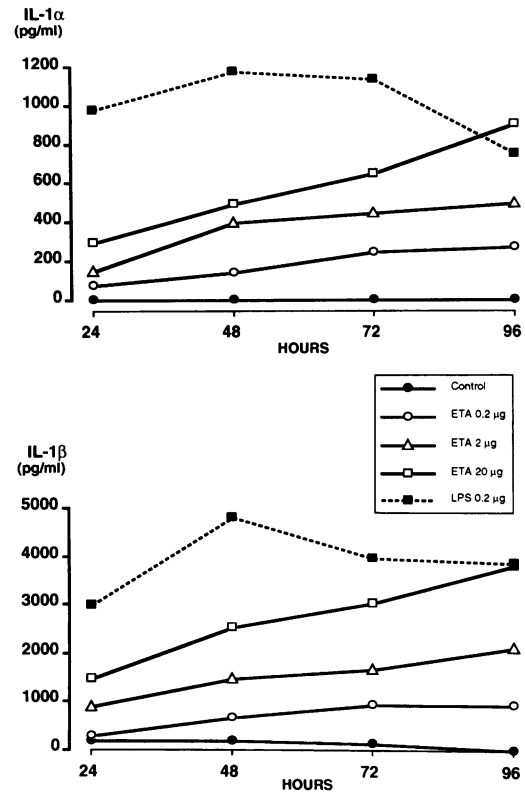

FIG. 1. Production of IL-1α and IL-1β by human PBMC stimulated with ETA and LPS, shown is an experiment representative of three others with cells from different donors.

(i) IL-1α and IL-1β release. Both IL-1α and IL-1β were released in a dose-dependent and time-dependent manner by the PBMC from all donors stimulated with ETA and LPS. The release was optimal after 48 h for the LPS-stimulated PBMC, whereas it progressively increased up to 96 h for ETA (Fig. 1). The amounts of the cytokines released by ETA-stimulated PBMC greatly varied from one donor to another. As little as 200 ng of ETA triggered the release of up to ca. 200 pg of IL-1α per ml and 800 pg of IL-1β per ml. For 20 μg of ETA per ml, the amounts released by 48 h ranged from 183 to 1,150 pg of IL-1α per ml and 2,552 to 5,802 pg of IL-1β per ml. Larger quantities were produced after 96 h of incubation. In contrast, these cytokines were practically not elicited by ETA-stimulated monocytes (Tables 1 and 2), whereas LPS triggered the release of significant amounts of both cytokines, ranging from ca. 300 to 450 pg of IL-1α per ml and 3,800 to 7,300 pg of IL-1β per ml depending on the donors. The assay for possible cell-associated IL-1α in ETA-stimulated monocytes was undertaken. The cells were collected, washed with RPMI medium resuspended in 0.5 ml of this fluid, and subjected to three cycles of freezing and thawing. Cell lysates were centrifuged, and the supernatant was assayed for IL-1α. No detectable amounts of this cytokine were found.

(ii) IL-6 release. No significant amounts of IL-6 were released by the monocytes after 48 h of incubation with ETA (Fig. 2). However, small amounts (90 to 220 U/ml) were released after 96 h of stimulation with 20 μg of ETA per ml. Under the same conditions, LPS triggered the release of an average amount of 1,000 U/ml (depending on donors) compared with controls (60 to 123 U/ml). In contrast to monocytes, much larger amounts of IL-6 were released in a dose-dependent and time-dependent manner by PBMC incubated with both stimulants (Fig. 2). IL-6 release by PBMC from seven different donors challenged with 20 μg of ETA per ml and 0.2 μg of LPS per ml ranged from 1,280 to 4,962 U/ml (mean, 2,437 U/ml) and 553 to 7,000 U/ml (mean, 3726 U/ml), respectively. As little as 200 ng of ETA elicited the release of ca. 500 U/ml.

(iii) TNF-α release. by ELISA and radioimmunoassay gave practically identical results with TNF-α. Both monocytes and PBMC stimulated with ETA and LPS released this cytokine. TNF-α elicitation by ETA-challenged monocytes was observed for the cells of 10 of 12 donors for the highest dose of toxin (20 μg/ml). After 24 h of incubation, TNF-α levels ranged from 796 to 1,196 pg/ml (mean, 990 pg/ml). LPS elicited moderately larger amounts (1,364 to 2,618 pg/ml; mean, 1,977 pg/ml) (Fig. 2).

Stimulation of PBMC from seven donors with ETA elicited the release of large amounts of TNF-α (Fig. 2) after 48 h of stimulation. The titers of this cytokine were almost the same for stimulation with 2 and 20 μg of ETA per ml. For the latter concentration, TNF-α release ranged from 5,652 to 14,340 pg/ml (mean, 10,671 pg/ml). Smaller amounts were elicited by LPS (1,336 to 9,740 pg/ml; mean, 5,915 pg/ml). The kinetic study of TNF-α production showed that the release of this cytokine was optimal after 24 to 48 h of incubation with LPS, 48 to 72 h for 20 μg of ETA per ml, and 96 h for the lower concentrations of toxin (Fig. 3). As little as 20 ng of ETA per

TABLE 1. Comparative cytokine-inducing capacity of ETA and ETC in human PBMC and mononuclear cells

Cytokine	Amt of cytokine[a] induced in:					
	Monocytes[b]			PBMC[b]		
	Control	ETA	ETC	Control	ETA	ETC
IL-1α	35	40	42	<30	808	1,063
IL-6	161	146	258	328	1,467	1,563
TNF-α	631	700	1,030	530	8,500	8,642
TNF-β	NT[c]	NT	NT	15	643	825

[a] Amounts are given in picograms per milliliter for IL-1α, TNF-α, and TNF-β and in units per milliliter for IL-6.
[b] Human monocytes were incubated for 24 h, human PBMC for 48 h in the presence of 20 μg of ETA per ml and 20 μg of ETC per ml. RPMI medium was used in control experiments. Results are from one of three representative experiments.
[c] NT, not tested.

TABLE 2. Comparative cytokine-inducing capacity of ETA, LPS, and heat-killed group A streptococci in human monocytes[a]

Cytokine	n[b]	Amt of cytokine[c] induced by:			
		Control[d]	ETA (20 μg/ml)	LPS (0.2 μg/ml)	Streptococci (20 μl/ml; 2×10^7 cfu)
IL-1α	6	<30	40 ± 10	374 ± 70	1,062 ± 151
IL-1β	3	280 ± 51	292 ± 56	5,562 ± 1,738	8,863 ± 1,036
IL-6	12	73 ± 6	111 ± 12	1,009 ± 278	1,218 ± 256
TNF-α	10	593 ± 34	1,032 ± 52	2,023 ± 183	12,070 ± 925

[a] Monocytes were incubated for 24 h at 37°C.
[b] n, number of experiments.
[c] Amounts are given in picograms per milliliter for IL-1α, IL-1β, and TNF-α and in units per milliliter for IL-6. Results represent the mean ± standard error of the mean.
[d] RPMI medium was used in control experiments.

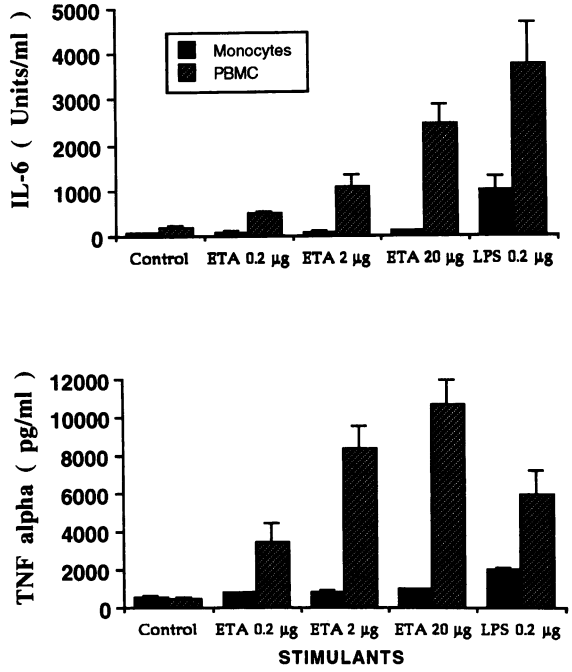

FIG. 2. Production of IL-6 and TNF-α by human monocytes and PBMC stimulated with ETA and LPS. The monocytes were cultured for 24 h and the PBMC were cultured for 48 h at 37°C. Data represent means ± standard errors of the mean for 12 independent experiments for monocytes and 7 independent experiments for PBMC. The doses indicated are in micrograms per milliliter.

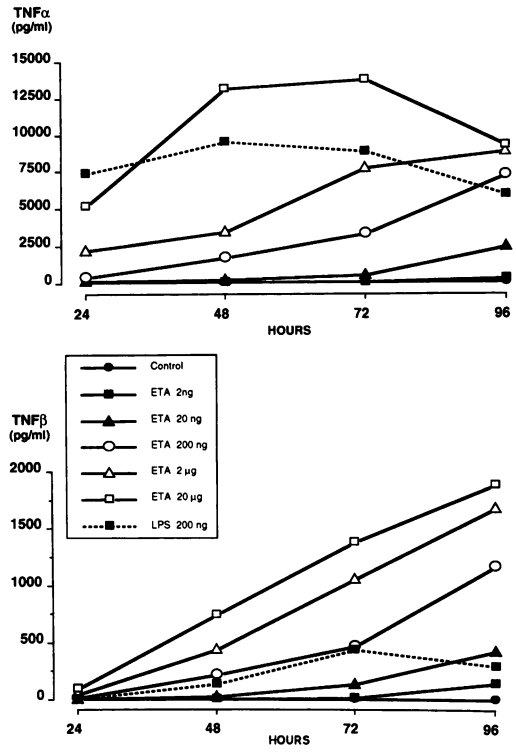

FIG. 3. Dose- and time-dependent production of TNF-α and TNF-β by PBMC stimulated with ETA and LPS. Shown is an experiment representative of three others with cells from different donors for both TNF-α and TNF-β.

ml elicited the release of ca. 2,500 pg/ml after 96 h of incubation, and 2 ng elicited the release of 1,000 pg/ml after the same period.

(iv) Effect of gamma interferon and IL-2 on TNF-α production by ETA-stimulated monocytes. Since only small amounts of TNF-α and IL-6 were elicited by ETA in monocytes, in contrast to the large amounts produced by PBMC, it appeared interesting to investigate whether the production of these cytokines could be influenced by adding gamma interferon or IL-2 (100 U/ml) to the monocytes challenged with ETA or LPS. IL-2 had no significant enhancing effect on cytokine release by either stimulant. Gamma interferon elicited a twofold increase of TNF-α production by ETA, whereas LPS-induced TNF-α production was ca. threefold enhanced (data not shown).

(v) TNF-β release. TNF-β was produced in a dose-dependent manner by PBMC incubated with various doses of ETA (Fig. 3). For the highest dose of ETA (20 μg/ml), the release of this cytokine ranged from 1,500 to ca. 2,000 pg/ml. As little as 2 ng/ml of toxin elicited the release of detectable amounts of TNF-β (154 pg/ml) after 96 h of incubation. Under the same conditions, only 200 to 500 pg of TNF-β per ml was obtained with LPS.

(vi) IL-8 release. IL-8 was elicited in a dose-dependent and time-dependent manner in PBMC stimulated by ETA or LPS (Fig. 4). The amounts of IL-8 produced after 72 h of incubation with the two stimulants ranged from 82 to 220 ng/ml for 20 μg of ETA per ml and 130 to 220 ng/ml for 0.2 μg of LPS per ml.

Cytokine-inducing capacity of ETC. ETC (20 μg/ml) was tested in parallel with ETA (20 μg/ml) for the induction of IL-1α, IL-6, TNF-α, and TNF-β by monocytes and PBMC. This toxin was at least as potent as ETA in the induction of these cytokines (Table 1).

Cytokine release by monocyte and PBMC suspensions stimulated with heat-killed streptococci. Streptococcal cell suspensions (ca. 10^9 CFU/ml in RMPI medium) were incubated for 24 h with 0.5 ml of monocyte suspensions (5×10^5 cells) and up to 96 h with 0.5 ml of PBMC suspensions (1.2×10^6 cells) from different donors (6 to 14 donors depending on experiments). For comparative purposes, the monocytes were stimulated in parallel with 20 μg of ETA per ml and 0.2 μg of LPS per ml, and the PBMC were stimulated in parallel with 5 μg of ETA or TSST-1 per ml and 0.2 μg of LPS per ml, respectively.

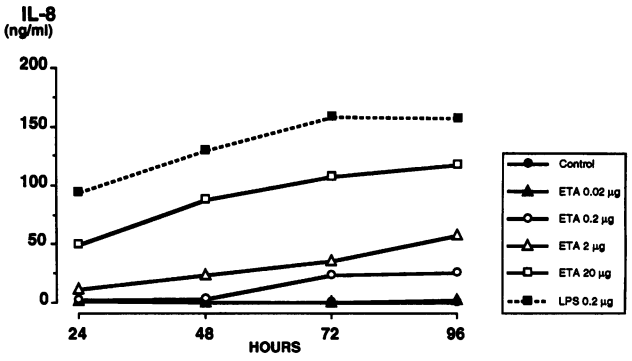

FIG. 4. Production of IL-8 by ETA- and LPS-stimulated human PBMC. Shown is an experiment representative of three others with cells from different donors.

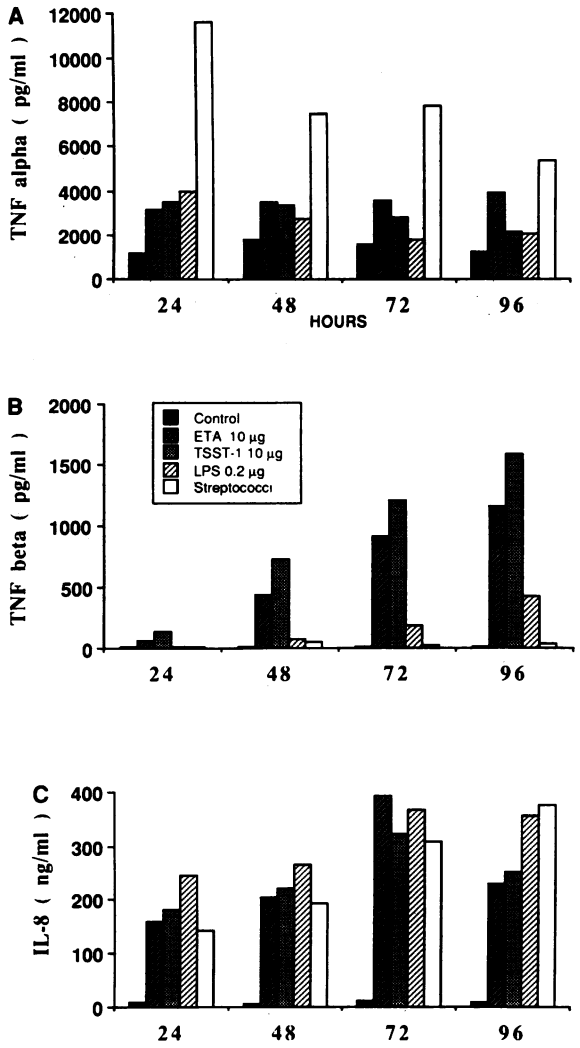

FIG. 5. Production of IL-8, TNF-α, and TNF-β by human PBMC stimulated with ETA, TSST-1, LPS, and heat-killed streptococci (20 μl/ml). Shown is an experiment representative of two others.

As shown in Table 2, in comparison with both ETA and LPS, streptococcal cells were very potent inducers of IL-1α, IL-1β, IL-6, and TNF-α in monocytes. Monocyte challenge with latex beads suspensions used as a control did not elicit cytokine release under the same conditions (data not shown). In accordance with the experiments reported above, poor release of these cytokines, except TNF-α, was elicited by ETA. A kinetic study of the release of IL-8 by PBMC incubated with the streptococci that very large amounts of this cytokine were produced, of the same order as the amounts triggered by ETA, TSST-1, and LPS (Fig. 5). TNF-α was also elicited in very large amounts by the PBMC-stimulated streptococci compared with ETA, TSST-1 and LPS under the same conditions. TNF-β was not induced by the bacteria. It was poorly elicited by LPS, whereas ETA and TSST-1 were very good inducers, the latter being slightly more potent.

DISCUSSION

The family of the polyclonal T-lymphocyte activators termed superantigens comprises a number of bacterial mitogenic proteins (26), including *Staphylococcus aureus* enterotoxins and TSST-1, *S. pyogenes* erythrogenic exotoxins (see Introduction), and other recently discovered streptococcal mitogens (6, 14, 29).

Several lines of experimental and clinical evidence strongly suggest that bacterial superantigens are causally involved in the pathogenesis of several acute and chronic human diseases (2, 6, 22, 26, 34, 36, 39), including STSS and TSS. These syndromes, as well as endotoxic shock, are thought to be due, at least in part, to the overproduction and release of various proinflammatory cytokines (particularly IL-1α, IL-1β, IL-6, IL-8, gamma interferon, TNF-α, and TNF-β) in response to patient monocytes/macrophage and T lymphocyte stimulation by *Staphylococcus aureus* and *S. pyogenes* superantigens (and possibly other exotoxins) or by gram-negative bacterial LPS. This contention is supported by the experimentally documented toxicity of unphysiological concentrations of many of these cytokines in animals (2, 31, 34) and by the elevated concentrations of some of these effectors (IL-1β, IL-6, IL-8, and TNF-α) in patient sera and (or) cerebrospinal fluid during TSS, STSS, and endotoxic septic shock (3, 5, 11, 20, 22, 27, 32, 34). Therefore, the study of the cytokine-inducing capacity of staphylococcal and streptococcal superantigenic exotoxins and LPS may greatly contribute to a better understanding of the molecular mechanisms of toxic shock pathogenesis and to the possible development of therapeutical strategies.

Extensive in vitro studies on the wide array of cytokines released by immune system cells stimulated with LPS, staphylococcal enterotoxins, and TSST-1 have been reported (see Introduction). In contrast, only limited investigations with ETs have been undertaken (3, 12, 21, 22). To our knowledge, the first evidence of the cytokine-inducing capacity of ETs was independently reported in two earlier works, showing the in vitro release of gamma interferon by immune system cells incubated with ETA (10, 40). The production of IL-2 and the expression of IL-2 receptor by ETA-stimulated human peripheral blood T lymphocytes was reported later, as was the monocyte-dependent polyclonal expansion of these lymphocytes (1), which had been observed earlier for ETA-challenged murine thymocytes (7). A few years later, the pivotal role of monocytes/macrophages via superantigen binding by major histocompatibility complex class II molecules proved a basic feature in the Vβ-dependent T-lymphocyte expansion which characterizes superantigens (2, 6, 24, 29, 36).

Several investigations in the past 5 years showed the release of TNF (12), TNF-α, IL-1β, and IL-6 (21) by human monocytes stimulated with ETA. The production of TNF-β by human PBMC stimulated with either ETA or TSST-1 was also reported (22). These effectors and others were also detected at the single-cell level by immunofluorescence staining with monoclonal antibodies on human monocytes and PBMC stimulated with ETA, enterotoxin A, and LPS (3).

In the present work, we assessed at the quantitative and kinetic levels the in vitro production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and TNF-β by human PBMC or monocytes after stimulation with ETA, ETC, LPS, and TSST-1. We also evaluated the effect of heat-killed group A streptococci on cytokine production to find whether *S. pyogenes* cells per se contribute via cytokine release to the pathogenesis of streptococcal TSS.

The response of PBMC and monocytes to ETA, ETC, and LPS showed both similarities to and divergences from the kinetics and amounts of cytokine release by these cells. At the optimal concentrations of ETA (20 μg/ml) and LPS (0.2 μg/ml), large amounts of IL-1α, IL-1β, IL-6, IL-8, and TNF-α were released by PBMC. ETA was almost twice as potent as

LPS in inducing TNF-α release. The level of this cytokine peaked at 10 ng/ml after 72 h of PBMC incubation with ETA. Even for as little as 2 ng of toxin, substantial amounts of TNF-α (ca. 1,000 pg/ml) were elicited. ETC behaved similarly to ETA, although the former proved slightly more potent. The detection and kinetic study of IL-8 release by PBMC in response to ETA and LPS is described here for the first time. This cytokine, which is a potent chemotactic factor and mediator of inflammation, plays an important role in the pathophysiology of sepsis in humans as inferred by the presence of high levels of circulating IL-8 in patients with shock (20, 27).

A striking difference between LPS and ETA (or ETC and TSST-1) in their cytokine-inducing properties in PBMC concerned the production of TNF-β. Only small amounts of this cytokine were elicited by LPS, in contrast to the high levels triggered by the superantigens. Similar results were reported for ETA and TSST-1 versus LPS (23, 24) and at the single-cell level in response to these toxins and enterotoxin A (3). TNF-β, also known as lymphotoxin-α, is produced strictly by T lymphocytes (13), whereas TNF-α is produced mainly by monocytes but also by T lymphocytes and other cells (28). These cytokines are involved in programmed cell death (apoptosis). Interestingly, a significant enhancement of apoptosis was elicited by in vitro stimulation with ETA of PBMC from patients infected with human immunodeficiency virus (19).

In monocytes, only small amounts of TNF-α and practically no IL-1α, IL-1β, and IL-6 were elicited by ETA and ETC, whereas large amounts of these cytokines were triggered by LPS. In contrast to monocytes, these cytokines were released in large amounts by ETA- and ETC-stimulated PBMC, suggesting a critical requirement for T lymphocytes in the induction by superantigens but not by LPS of these essentially monocyte-derived cytokines. Furthermore, the addition of exogeneous IL-2 and gamma interferon to the toxin-challenged monocytes failed individually or in combination to substitute for the signal delivered to these cells by T lymphocytes, indicating the necessity for a direct cell-cell contact (13, 17) without excluding the contribution of soluble factors (37). T-cell involvement was similarly required for cytokine release by mouse monocytes in response to ETA. This superantigen induced small amounts of IL-6 in BALB/c mouse peritoneal macrophages and large amounts of IL-6 and IL-3 in BALB/c and C3H/HeJ mouse splenocytes. Nude BALB/c mice splenocytes stimulated with ETA did not produce these cytokines (30).

Similarly to ETA and ETC, T-cell help was also required for the release of IL-1 and TNF-α in response to *Staphylococcus aureus* enterotoxins and TSST-1 but not in response to LPS (28, 40). The central role of T lymphocytes and particularly TNF release in lethal shock pathogenesis triggered by *Staphylococcus aureus* enterotoxin B was also shown to occur in mice (18, 28).

The kinetics of cytokine release by PBMC in response to LPS and ETA differed significantly. With the former, the release of IL-1α, IL-6, and TNF-α was almost optimal after 24 h of incubation and that of IL-1β was optimal between 24 and 48 h, whereas IL-8 and TNF-β levels peaked at 72 to 96 h. A delayed and gradual cytokine release occurred with ETA. Levels of almost all cytokines peaked between 72 and 96 h, except for that of TNF-α, which was practically optimal at 24 h. Similar lower rates of cytokine release by PBMC stimulated with ETA, TSST-1, and enterotoxin A, compared with the rapid production triggered by LPS, were reported (3). This delay in cytokine release in response to the superantigens might be due to the prerequisite time-dependent T-cell activation of the monocytes by these toxins. Consequently, the two different patterns and dynamics of cytokine release by LPS and by superantigens may reflect some of the differences in the clinical features of toxic shocks provoked by gram-negative bacteria versus *Staphylococcus aureus* and *S. pyogenes*.

Significant interindividual differences in the levels of the cytokines released by target cells in response to ETA, ETC, and LPS were observed, as also reported for these effectors and for *Staphylococcus aureus* superantigens by other investigators (3). The quantitative differences in cytokine production may explain the various degrees of severity of toxic shock among patients; they may also reflect variations in the expression, number, and availability of the relevant Vβ chain motifs of T-cell receptors on patient lymphocytes and possibly differences in superantigen affinity toward these motifs and/or major histocompatibility complex class II molecules (24).

The study of various *S. pyogenes* strains isolated from patients with STSS and lacking the structural genes of ETA and ETC (35) allowed us to identify a novel cytokine-inducing superantigen which potently stimulated T lymphocytes bearing the Vβ 4, 7, and 8 motifs (14). This product and that recently described by Mollick et al. (29) may contribute, together with ETs, to the induction of STSS in infected patients by strains producing these newly discovered superantigens.

The potential cytokine-inducing capacity of heat-killed group A streptococci on human monocytes and PBMC was also investigated in this work. After 24 h of incubation of monocytes with these microorganisms, large amounts of IL-1α, IL-1β, IL-6, IL-8, and TNF-α were released. They exceeded those elicited by ETA and LPS in these cells and in PBMC except for IL-6. TNF-α release peaked at 24 h, indicating a rapid cell reaction to bacterial challenge. In contrast to ETA or ETC, streptococcal cells failed to induce TNF-β. This finding constitutes a major difference between *S. pyogenes* superantigens and streptococcal cells in their cytokine-inducing capacity. Invading bacteria are, prior to exotoxin release, the first elements which interact with the immune system cells of the infected host. In the case of bacterial resistance to phagocytosis, which leads to a reduced contact of the bacteria with immune cells, the superantigens may subsequently trigger another cytokine pathway and induce shock. The failure of latex beads to trigger cytokine release in vitro by monocytes, in contrast to the heat-killed streptococci, indicates that cytokine production in this context was linked to a specific process inherent to streptococci par se but not to particle phagocytosis.

The present work supports the contention that the high levels of the cytokines released by target cells stimulated with streptococcal superantigens (ETA, ETC, and other exoproteins) and streptococci are major mediators of STSS. The main difference between the effects of LPS and superantigen observed in this work concerns the induction of TNF-β. This cytokine therefore appears to be a characteristic mediator involved in the toxic shock induced by ETs, as also suggested by other investigators (3, 22), whereas TNF-α is a common endogeneous mediator in the shock elicited by both gram-negative bacterial LPS and *S. pyogenes* or *Staphylococcus aureus* superantigens (5, 9, 12, 22, 34, 36).

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