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Dialysable leucocyte extract (DLE) reduces lipopolysaccharide- induced tumour necrosis factor secretion in human leucocytes.

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Abstract

Dialysable leucocyte extract (DLE), obtained from lysed leucocytes, provide clinical effectiveness in a broad spectrum of diseases. Tumour necrosis factor (TNF) is raised in AIDS patients leading to an increase in human immunodeficiency virus (HIV) replication in vitro [1,2], whereas progression to AIDS in asymptomatic HIV infected individuals is retarded under treatment with DLE. In the present study we tested the DLE effect in vitro on both TNF biological activity (cytotoxicity) in L929 cells and its induction by lipopolysaccharide (LPS) in human monocytes as well as in whole blood from healthy donors. When monocytic cells were simultaneously exposed to LPS and DLE during a period of $5\frac{1}{2}$ hours, the induction of TNF was strongly diminished. The same inhibitory effect of DLE on TNF induction was observed when LPS was added to the culture medium prior to DLE. No significant effect of DLE on TNF-mediated cytotoxicity, even in the presence of the highest concentrations of DLE tested, was detected. DLE treatment of whole human blood regulates responses to LPS: simultaneous in vitro exposure to endotoxin provokes a remarkable decrease (4- and 1.6-fold) of TNF release. In pre-incubation experiments, TNF production was largely reduced or completed abrogated. These results could, in part, explain the in vivo observed effect, when under treatment with this extract, the progression to AIDS of HIV-infected individuals was retarded. The results suggest that 'natural' substances like DLE may be important immunomodulators in inflammatory diseases.

Abbreviations: DLE: dialysable leucocyte extract; HIV: human immunodeficiency virus; hrTNFα: human recombinant tumour necrosis factor α; IFNα: interferon α; LBP: LPS-binding protein; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells; TNF: tumour necrosis factor.

Introduction

Activated phagocytes synthesize various cytokines which can influence cellular systems via a cascade of events culminating in an inflammatory response. In particular, TNFα, mainly produced by activated monocyte/macrophages, has a broad spectrum of biological activities, including a major role in immune and inflammatory responses. The importance of TNF, as an early response mediator in inflammation, seems to be associated with the magnitude of its expression in vivo. The exaggerated production of TNF results in physiopathologic responses and the generation of disease associated symptoms. This cytokine has been implicated in pathogenic mechanisms of inflammation, cachexia, tissue injury, septic shock and multiple organ failure [3–5], and its increased production has been associated with high morbidity and mortality rates. HIV-related disorders enhance serum levels of TNFα and it is known that TNFα is able to increase HIV replication in vitro [6,7]. Furthermore, TNF seems to mediate pathogenic mechanisms in other non-infectious disorders, including rheumatoid arthritis, systemic lupus erythematous, bowel necrosis and multiple sclerosis [8].

DLE is a biological extract with widespread effects on the immune system. It is derived from immune lymphocytes and is capable of transferring specific immunity to naive T-lymphocytes. Since cell mediated immunity plays an important role in the control of infection, transfer factor has been used in the treatment of a broad spectrum of viral, parasitic and fungal dis-

eases [9]. Its therapeutic effects have been reported in the treatment of patients with septic shock and related diseases [10]. We have observed a remarkable response in early stages of HIV infection: progression to AIDS in asymptomatic HIV-infected individuals was significantly retarded with DLE treatment in a 6-year clinical trial (manuscript in preparation).

The afore-mentioned pathogenic role of TNF in these disorders prompted us to explore whether the observed effects of DLE treatment could be, at least partially, associated with a regulated TNF response. The experiments presented herein evaluate the DLE effect in vitro on both TNFα biological activity and induction of this cytokine by LPS-stimulated human monocytes. Our results demonstrate that in vitro DLE treatment reduces TNF production by human peripheral blood leucocytes activated with LPS.

Materials and methods

Cell Cultures

The murine fibroblast cell line L929 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere. Human peripheral blood mononuclear cells were isolated from buffy coats of healthy donors by Ficoll-Paque density centrifugation (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) and were cultured in RPMI 1640 medium supplemented with 10% FCS.

Reagents

LPS derived from E. coli 0111:B4 was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human Tumour Necrosis Factor α (hrTNFα) with a specific activity of $2x10^8$ U/mg was obtained from Boehringer Mannheim. Dialysable Leucocyte Extract was produced (Biological Research Center, La Habana, Cuba) using pooled leucocytes from healthy donors that were previously induced with Sendai virus to produce Interferon α (IFNα). The dialysable leucocyte preparation contains entities with molecular weights under 10000 Da. IFNα activity was undetected in DLE preparations when tested by means of inhibition of virus cytopathic effect. One unit was defined as the dialysable material derived from $5x10^8$ leucocytes. DLE preparations were tested for contamination with endotoxin by the Limulus amoebocyte lysate test (Chromogenix, Mölndal, Sweden). The endotoxin content was <10pg/ml.

Induction assay on human monocytes

PBMC cultures were resuspended at a concentration of $1x\ 10^7$ cells/ml and 100μl were transferred to 96-wells flat-bottom tissue culture plates (Nunc, Denmark). Cells were incubated in a 5% CO_2 atmosphere at 37°C for 2 hours in order to isolate monocytes by adherence. After removal of nonadherent cells by aspiration, the monolayer of monocytes was extensively washed with cold PBS and then stimulated with LPS in serum-free medium during a period of $5\frac{1}{2}$ hours. The dose of LPS and the period of incubation for the optimum TNF production were determined in preliminary experiments. TNF production was maximal after incubation of monocytes with 10–20μg/ml LPS. Two assay conditions were performed: simultaneous incubation of monocytes with LPS and DLE, and treatment of cells with DLE 1 hour after induction with LPS. DLE concentrations tested in both experiments were 0.3; 0.6 and 1.25 U/ml. After incubation, supernatants were collected to determine TNF concentrations.

L929 TNF bioassay

The murine fibroblast cell line L929 was used in the cytotoxicity assay as previously described [11]. Monolayer cultures were maintained in RPMI medium supplemented with 10% of FCS. 100μl, containing $1x\ 10^5$ L929 cells, were added to each well of a 96-well, flat-bottom plate (Nunc, Denmark) and cultured overnight at 37°C in a 5% CO_2 atmosphere. After this period of incubation, 50μl of Actinomycine D (4 μg/ml) and 50μl of different concentrations of hrTNFα or 50μl of monocytic supernatants were added to the culture plate. Fifty μl of mixtures containing different concentrations of DLE plus hrTNFα were added per well, in experiments testing DLE effect on L929 cell lytic assay. Following 24 hours of incubation at 37°C, cytotoxicity was visualized by crystal violet staining and measured with a plate reader at 540nm.

Whole blood TNF assay

The induction of TNF by LPS in whole blood was measured according to Warren et al. [12]. Briefly, heparinized blood from healthy donors was diluted 1:5 in RPMI 1640 culture medium. 50μl/well diluted blood were seeded in plastic culture dishes (24 wells;

Nunc, Denmark). The cultures were always maintained in a 5% atmosphere at 37°C. Two assay conditions were performed: simultaneous incubation of cells with LPS (2 or 20 ng/ml) and DLE (0.125–2 U/ml) and pretreatment with DLE for 2 hours prior induction. In both cases the assay was carried out during four hours. TNFα was measured in culture supernatants using a specific ELISA system kindly provided by Dr. W. Buurman from the University Hospital, Maastricht. Briefly, plates were coated overnight at 4°C with 100μl/well of a murine monoclonal anti-human TNFα antibody (61E71) in PBS. Then, wells were blocked with 125μl/well of PBS and 1% BSA during 1 hour at room temperature. Recombinant human TNFα was used as a standard by diluting in PBS containing 0.1% BSA. Cell supernatants (100μl/well) were incubated for 3 hours at room temperature. After emptying plates, 100μl/well of a rabbit polyclonal anti-human TNFα diluted in PBS 0.1% BSA was added and incubated for 1 hour at room temperature. This was followed by addition of a peroxidase-labelled goat anti-rabbit Ig conjugate (Sigma Chemical Co., St. Louis, MO) diluted in PBS 0.1% BSA for 1 hour at room temperature. After washing with 0.1% Tween–20 in distilled water, 100μl of the tetramethylbenzidine peroxidase substrate (1mg/ml) prepared in peroxidase solution (citrate buffer 0.05 M, pH 5.0) was added to each well. The reaction was stopped by addition of 100μl/well of 1M H_2SO_4. The absorbance was measured in a plate reader at 492 nm. The sensitivity of this assay was 5 pg/ml. The ELISA was specific for TNFα and did not react with other cytokines.

Results

Effect of DLE on TNF cytotoxicity

We were interested in testing the potential direct effect of DLE treatment on L929 cells, regarding cell sensitivity to the cytotoxicity mediated by TNF-α. To evaluate this effect of DLE on the TNF bioassay we selected TNF doses able to produce in our experimental model between 45–70% of maximal cytotoxicity. TNF concentrations ranging from 0.20–0.45 ng/ml ensured a significant cytotoxicity after 24 h of treatment of cell monolayers (data not shown). The effect of DLE on TNF mediated cytotoxicity on L929 fibroblasts was evaluated using non-toxic DLE concentrations ranging from 0.15 to 2.5 U/ml. The assay was performed by simultaneous incubation of cell monolayers with

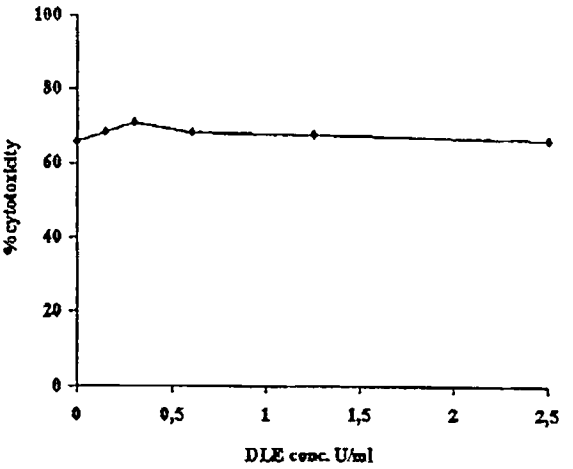

Figure 1. Evaluation of DLE effect in the cytotoxicity assay. Confluents L929 cells were exposed to TNFα and DLE simultaneously. For this experience cells were incubated with TNF (0.3 ng/ml) and increasing doses of DLE ranging from 0.15 to 2.5 U/ml during 24h.

TNFα and DLE during 24 hours. We did not detect a significant effect of DLE treatment on TNF-mediated cytotoxicity, even in presence of the highest concentrations of DLE tested. Figure 1 shows a representative experiment demonstrating that the selected range of DLE doses do not modify cytotoxicity mediated by a fixed TNFα concentration.

DLE modulation of LPS-induced TNF production by isolated monocytic cells

The effect of DLE on endotoxin-induced TNF production by isolated human monocytes was examined by incubating cultures with high concentrations of E.coli LPS (10–20μg/ml) in serum free-medium (therefore lacking LBP). Freshly isolated human monocytes were sensitive to increasing doses of LPS in serum absence, producing correlative increments in TNF production. According to our results, simultaneous exposure of human monocytes to LPS and DLE during $5\frac{1}{2}$ hours decreased TNF release induced by LPS (Fig. 2A). Regardless the LPS stimulating concentration, an inhibitory effect was observed. Mean levels of cytokine secretion for three donors demonstrated more than 2.5-fold reduction in TNF secreted by monocytes treated with DLE (0.3–1.25 U/ml).

To explore the influence of DLE treatment on the reversion of the activated state, experiments were conducted on cells already stimulated with LPS. It is noteworthy that the addition of DLE after 1 hour of activation with LPS produced a significant inhibitory effect

on TNF release. In figure 2B are shown the results from an experiment in which the inductive effect of LPS (10 ug/ml) was markedly reduced. TNF release was affected in a lesser extent at 20 ug/ml LPS (1.5–2.3 fold; Fig. 2B). It is important to note that TNF mean-secretion levels from three other donors, also evidenced a significant inhibition, after DLE post-activation treatment.

DLE effect on LPS-induced TNF release in wholeblood

The preceding experiments indicate that DLE reduces monocyte LPS-induced TNF release, when using experimental conditions where commonly involved serum proteins are very diluted or absent, and where cells were exposed to very high concentrations of endotoxin. To further explore if DLE treatment regulates responses to physiologically relevant doses of LPS, a model system comprising a full complement of soluble components and cytokine secreting cells was used: LPS-stimulated TNF release from leucocytes was evaluated in whole human blood.

Simultaneous in vitro treatment of human leucocytes with DLE 2 U/ml or 0.125 U/ml and LPS (2 ng/ml) reduced 4- and 1.6-fold maximal TNF production measured 4 hours after induction respectively (Fig.3A). Under these experimental conditions, the addition of minimal DLE dose determined TNF secretion at levels only slightly higher than those observed in LPS absence (74.3±3.2% inhibition of induced levels). Treatment with 2 U/ml of DLE completely blocked the LPS- induced TNF release, producing cytokine quantities even lower than in LPS-free cultures.

To determine if pre-exposition of leucocytes to DLE additionally enhances the observed inhibitory effect, 5-fold diluted human blood was incubated with various DLE doses (0.125–2 U/ml) 2 hours prior LPS (2 or 20 ng/ml) addition. In this assay mimicking a natural environment, TNF production in response to endotoxin was greatly reduced (DLE 0.125–0.5 U/ml) or completely abrogated (1–2 U/ml) using LPS concentrations 10-fold higher than those used for simultaneous treatment (Fig. 3B). It is also remarkable in these ex vivo experiments that TNF release was consistently decreased below the observed minimal levels when only DLE was added to whole human blood (Fig. 3).

Discussion

Uncontrolled and over exuberant cytokine production constitutes a central pathogenic mechanism in systemic inflammatory responses triggered by LPS from gram-negative bacteria. Early events associated with injurious responses to endotoxin are attributable to upregulated secretion of TNF by activated monocyte/macrophages and other cell types. Human monocytes are exquisitely sensitive to LPS, and levels as low as 10 pg/ml can induce the rapid synthesis of inflammatory cytokines as TNFα and IL–1ß [13].

Although we did not detect a significant effect of DLE treatment on TNF-mediated cytotoxicity, in this study we demonstrated that the exposure of human leucocytes to DLE greatly reduces the secretion of TNF active protein triggered by LPS. The inhibitory effect of DLE was observed both in cultures of isolated mononuclear cells and whole blood experiments.

High concentrations of LPS were required to activate TNF release from human isolated monocytes under serum-free conditions. This is in agreement with several reports demonstrating that washed human phagocytic leucocytes respond with reduced sensitivity to LPS in the absence of serum [14,15]. DLE was able to downregulate secreted TNF amounts measured at $5\frac{1}{2}$ hours after high dose LPS challenge. The inhibition by DLE in the absence of serum proteins was not overcome by increasing concentrations of LPS. Therefore, DLE- mediated TNF reduced secretion seems not to be dependent on molecules competing with LPS at specific cellular recognition sites. It is widely accepted that LPS activation of leucocytes mainly occurs as a result of the interaction of LPS-LBP complexes (or LPS complexes with other serum proteins) with CD14, a glycosyl phosphatidylinositol- linked protein expressed on the surface of monocytes, macrophages [16] and polymorphonuclear leucocytes [15,17]. While this CD14-dependent pathway predominates at low concentrations of LPS, at higher concentrations and in the absence of serum accessory proteins, a CD14-independent pathway appears to predominate [14]. In our free-serum experiments it is likely that activation primarily occurs by the former mechanism, although minor stimulating events through CD14-LPS direct interaction or even the presence of minimal amounts of absorbed serum accessory proteins could not be excluded. Thus, our observations of partial but significant inhibition in free-serum conditions should not involve mechanisms mediated by LBP, septin or soluble CD14.

It was remarkable that DLE, added to monocyte cultures after 1 hour of LPS stimulation, reduces TNF secretion to levels similar to those obtained under simultaneous incubation. LPS induces very

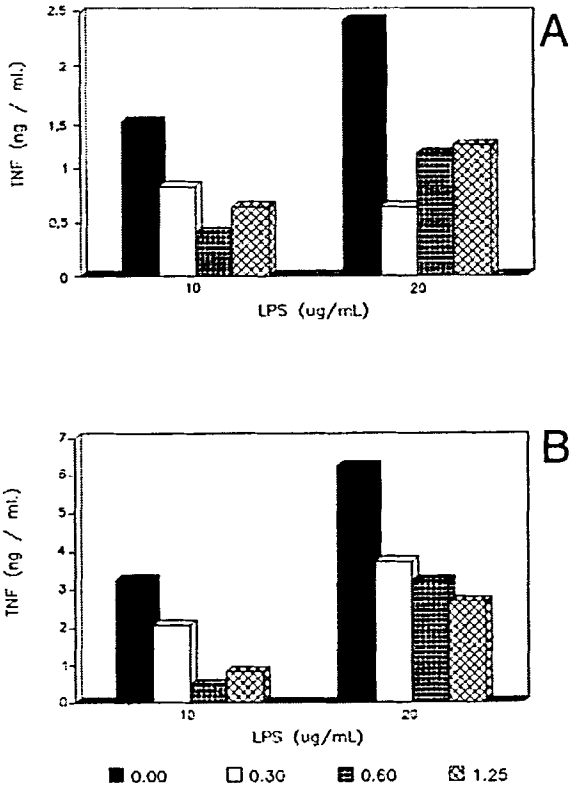

Figure 2. Effect of DLE on monocyte TNF release induced by LPS. Isolated monocytic cells were treated with 10 or 20μg/ml of endotoxin and various concentrations of DLE in serum-free conditions. DLE was added to cell cultures both simultaneously (panel A) or 1 hour after LPS addition (panel B). Cell supernatants were harvested after 5:30 h and titrated by bioassay for TNF-α. Results shown are from a single representative experiment, and similar inhibition patterns were seen on monocytic cells isolated from three different healthy volunteers. TNF-α secreted amounts in absence of LPS were 213 pg/ml and 425 pg/ml for experiments shown in panel A and B respectively.

rapid upregulation of inflammatory gene transcription, increases the stability of the message and also enhances protein expression at the translational level [18, 19]. TNF mRNA expression peaked after 0.5–2 hours of bolus LPS addition and then declined [20, 21], with protein maximal expression at 4–8 hours. In addition, previous studies have suggested that monocytes become committed to express TNFα after only 5- to 15 min exposure to LPS [22]. Our results could rather indicate that LPS-induced post-transcriptional mechanisms are impaired by DLE treatment, but its influence on gene transcription regulation can not be ruled out. Additional experiments must be carried out to determine the site(s) or event(s) of inhibition. Others have reported inhibition of TNF expression by blocking second messenger pathways [23] which mediate not only a fast TNFα mRNA accumulation, but also induce a rapid TNF production [24].

Since results from studies on the response to LPS with isolated monocytes can be complex due to facts like partial cell activation by isolation procedures, the absence of cell and plasma components involved in physiological responses, and the use of much higher LPS concentrations than those typically found in the blood of septic patients [25, 26], we decided to explore the effect of DLE treatment on whole human blood. Simultaneous in vitro treatment of human leucocytes with DLE and LPS strongly reduced maximal TNF production, measured 4 hours after induction. Actually high dose DLE treatment blocked the release of TNF-α induced by LPS and only slight increases over baseline levels were observed in leucocytes treated with low dose DLE. This strong inhibitory response in a sys-

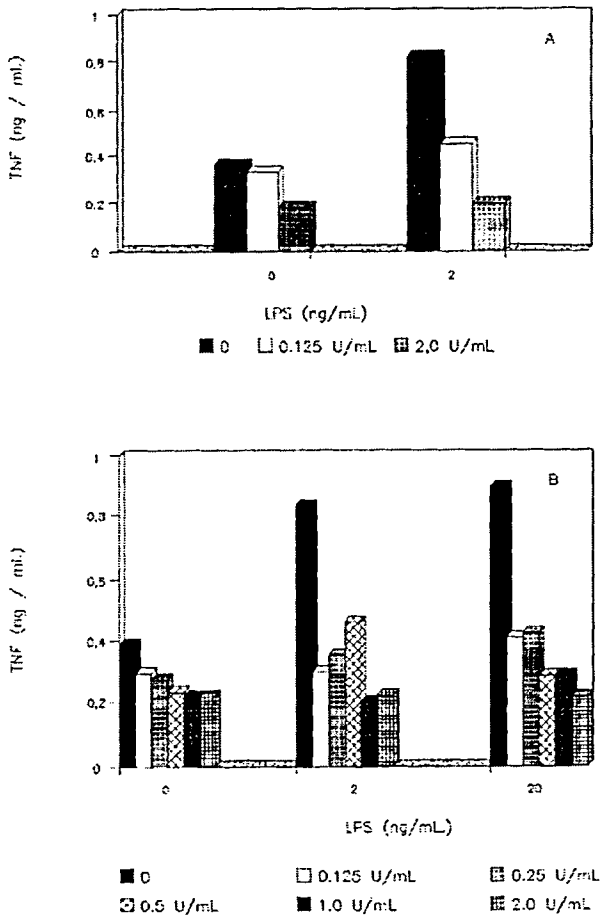

Figure 3. Inhibitory effect of DLE on LPS-induced TNF release in whole human blood. (3A) 500μl aliquots of whole blood were treated simultaneously with LPS (2 ng/ml) and two different concentrations of DLE (0.125 and 2 U/ml), or (3B) stimulated with LPS (2 and 20 ng/ml) after 2 hours of treatment with various DLE doses (0.125–2 U/ml). Cultures maintained only in presence of LPS or free of added LPS were included as maximal and basal controls of TNF release respectively. Supernatants were harvested 4 hours after LPS addition and used to quantify TNF production using ELISA. The result are representative of three separate experiments using blood form different donors. TNF secretion base line levels for polimixin B (25μg/ml) treated leucocytes was 375 pg/ml and 398 pg/ml in experiments A and B respectively.

tem that mimics physiological conditions emphasizes the therapeutic potentiality of DLE in LPS associated inflammatory diseases. Reduced TNF production following induction with low dose LPS seems to indicate that DLE treatment affects the CD14-dependent pathway of LPS-stimulated cytokine secretion, or that a common event triggered by LPS interaction with CD14 or other cell receptor must be the target of DLE inhibition. An LPS-induced post-transcriptional mechanism could be the site of inhibition. Again further data are necessary to assert any proposition. The full cell component in whole blood, including polymorphonuclear cells that can produce TNF in response to LPS [27], demonstrated that DLE affected responses of different cell types. An increase in the LPS- neutralizing capability of plasma should also be considered in future experiments.

The pre-treatment of leucocytes in whole blood with DLE offered no significant differences in the observed inhibitory effect. Others have reported that the exposure of monocyte cultures to DLE, 3 hours prior to induction, enhances TNF production [28]. Those studies were carried out with isolated macrophages in presence of serum, making comparisons difficult. In

addition, variable composition of DLE could explain dissimilar results between the two studies. Indeed, the obtention of standardized active material is until now an unresolved problem.

TNF production induced by LPS has long been suspected to play an important role in septic shock. Given the observation made in this study that DLE leucocyte treatment could inhibit or abrogate LPS-induced TNF release, and substantially reduce TNF production by previous activated cells, the therapeutic use of DLE in gram-negative sepsis should be further explored. Reduced C Reactive Protein concentrations in septic patients after treatment with DLE have been previously reported [10]. This in vivo response could be at least partially dependent on the control of TNF secretion. The elucidation of mechanisms mediating the DLE inhibitory effect requires additional studies. Results from these studies could have also particular relevance for the treatment of other very dissimilar diseases, where TNF secretion plays a pathogenic role, including asthma, rheumatoid arthritis, AIDS and schistosomiasis [8,29,30].

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