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Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells

J. S. FRIEDLAND, R. SHATTOCK, D. G. REMICK* & G. E. GRIFFIN *Division of Communicable Diseases, St George's Hospital Medical School, London, UK, and *Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA*

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SUMMARY

Monocytes having phagocytosed mycobacteria are known to present the bacterial 65-kD heat shock protein (hsp) on their cell surface to $\alpha\beta$ and $\gamma\delta$ T lymphocytes. Cytotoxic CD4$^+$ cells may then lyse monocytes expressing mycobacterial 65-kD hsp. However, it is not known whether 65-kD hsp directly stimulates monocyte functions other than antigen presentation. This study has demonstrated that following extraction of bacterial lipopolysaccharide, purified recombinant mycobacterial 65-kD hsp may directly activate THP-1 cells, a human monocytic line, to accumulate mRNA for and secrete tumour necrosis factor (TNF), a cytokine important in granuloma formation, the characteristic host immune response to mycobacterial infection. TNF gene expression and secretion following stimulation by hsp was dose-dependent and abolished by heat-induced proteolysis. Subsequently, THP-1 cells secreted IL-6 and IL-8, cytokines involved in recruitment and differentiation of T lymphocytes. The data indicate that secretion of proinflammatory cytokines from monocytes activated by mycobacterial 65-kD hsp may be important in the host immune response and in the development of antigen-specific T cell-mediated immunity.

Keywords heat shock protein cytokines monocytes

INTRODUCTION

Phagocytosis of mycobacteria by monocytes is followed by presentation of mycobacterial 65-kD heat shock protein (hsp) on the monocyte surface in association with MHC class II molecules. Mycobacterial 65-kD hsp is recognized by $\alpha\beta$ T cells [1] and $\gamma\delta$ T cells [2]. Cytotoxic CD4$^+$ lymphocytes are capable of lysing monocytes expressing mycobacterial 65-kD hsp [3], although within the activated monocyte up-regulation of 65-kD hsp may protect intracellular microorganisms from superoxides and other noxious molecules [4]. However, it is not known whether 65-kD hsp exerts any direct effect upon human monocytes such as stimulating release of proinflammatory cytokines, involved in both early cellular antimycobacterial immune responses and in recruitment of antigen-specific T cells. Such a phenomenon might have great implications for the host response to mycobacterial infection, since cooperation between cells of monocyte lineage and T lymphocytes is crucial to the formation of granuloma, important in restricting replication of mycobacteria [5].

Cytokines secreted by monocytes and macrophages are involved in killing of intracellular microorganisms, possibly acting via induction of nitric oxide [6]. Both tumour necrosis factor (TNF) and IL-6 have antimycobacterial activity in murine bone marrow-derived macrophages [7,8]. These proinflammatory cytokines are known to be secreted following phagocytosis of *Mycobacterium tuberculosis* ([9] and Friedland *et al.*, unpublished observations).

Monocyte-derived cytokines, such as TNF, are also pivotal in granuloma formation [10]. Monoclonal anti-TNF prevented granuloma formation and allowed unchecked multiplication of *M. bovis* (BCG strain) in a mouse model [11]. TNF may be particularly important in the maintenance of granuloma [12]. In addition to cells of the monocyte lineage, T cells are involved in granuloma formation. Athymic rodents are usually unable to mount any granulomatous response [5]. Both IL-6 and IL-8 are monocyte-derived cytokines which modulate T lymphocyte functions. T lymphocytes express high affinity IL-6 receptors [13] and IL-6 stimulates T cell proliferation [14]. Recruitment of T lymphocytes to granuloma probably depends in part on secretion of IL-8, a T cell chemoattractant, from monocytes [15]. IL-8 is released in extremely high concentrations by monocytic cells that have phagocytosed *M. tuberculosis* [16]. However, although TNF, IL-6 and IL-8 may be involved in T cell activation and formation of granuloma, many other stimuli including direct cell–cell interactions involving adhesion molecules are also important.

Correspondence: Dr Jon S. Friedland, Division of Communicable Diseases, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

The present study was designed to investigate whether gene expression and secretion of proinflammatory cytokines occurred in THP-1 cells, a human monocyte cell line [17], in response to stimulation by purified *M. leprae* 65-kD hsp. This protein is over 95% homologous to the analogous *M. tuberculosis* and *M. bovis* hsp [18]. We investigated release of TNF, IL-6 and IL-8 from THP-1 cells as these cytokines have been demonstrated to be involved in the monocyte response to mycobacteria and in recruitment and activation of antigen-specific T cells.

MATERIALS AND METHODS

Cell culture

THP-1 cells (American Type Culture Collection, TIB 204), a human monocytic cell line, were suspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS; endotoxin-free), 2 mM glutamine and 1% penicillin/gentamicin (all Sigma, Poole, UK). Cells (10^7) were cultured in a 5-ml volume at 37°C in 5% CO_2 in PTFE vials (Pierce, UK) to prevent adherence of monocytes and consequent cytokine secretion [19]. Purified 65-kD hsp was added to cultures ($t=0$) and at each time point all cells from a single vial were pelleted by centrifugation at 1000 *g* for 5 min.

65-kD hsp preparation

Recombinant 65-kD hsp from *M. leprae* [20] (the generous gift of Dr M. J. Colston, National Institute for Medical Research, London, UK) was passed three times through a Detoxigel column (Pierce) at 4°C in order to remove any bacterial lipopolysaccharide (LPS). Following column purification, hsp protein was shown to have remained intact by SDS–PAGE gel electrophoresis and quantified using the Bradford method [21]. Removal of LPS from hsp was confirmed by measurement using the limulus amoebocyte assay (kindly performed by Dr S. Poole, National Institute for Biological Standards and Control, South Mimms, UK). This method was used in preference to addition of polymixin directly to experiments as it allowed both determination of protein stability and quantification of any residual LPS contamination.

Cytokine analysis

Cell culture supernatant was assayed for TNF using the WEHI 164 cell line, subclone 13 [22]. IL-6 was assayed using the B9 cell line proliferation assay [23] which we have previously shown to be specific for this cytokine [24]. IL-8 concentrations were measured with a previously described ELISA [25].

RNA extraction and Northern blotting

Cell pellets were homogenized in 4 M guanidine thiocyanate, 25 mM Tris pH 7·0, 0·5% N-lauroylsarcosine and 0·1 M 2-mercaptoethanol. Following double phenol-chloroform and chloroform-isoamyl alcohol extraction, precipitation in isopropanol and then in ethanol, RNA was suspended in DEPC water. After quantification on an optical densitometer (Pye/Unicam SP6-450), 15 μg RNA aliquots were run on formaldehyde 1% agarose gels, transferred by capillary blotting to Hybond-N (Amersham International, Amersham, UK) and fixed with ultraviolet light.

Hybridization and oligonucleotide probing

Northern blots were prehybridized with 6× SSC, 1× Denhardt's, 0·5% SDS, 0·05% sodium pyrophosphate, 50 μg/ml polyadenylic acid and 100 μg/ml transfer RNA. Subsequent hybridization used γ-^{32}P 5′ end-labelled oligonucleotide probes; a 25 mer for TNF, 30 mers for IL-6 and IL-8 [26] and a 40 mer for β-actin [27]. Northern blots were then autoradiographed with intensifying screens at −70°C. Between probings, blots were stripped by heating for 1 h at 65°C in a solution of 0·005 M Tris-HCl pH 8·0, 0·002 M Na_2 EDTA and 0·1× Denhardt's. Autoradiographs were scanned using a Scanjet Plus (Hewlett Packard, CO) into a Macintosh IIsi computer (Apple Computer, CA) and data quantified with image 1·41 (from National Institutes of Health Research Services Branch, USA).

RESULTS

TNF gene expression and secretion

THP-1 cells were stimulated by purified 65-kD hsp (10 μg/10^7 cells containing < 1 ng LPS/ml culture medium) and TNF mRNA generation quantified at 90 min. We have shown in previous experiments that this time coincides with maximum accumulation of this mRNA species in THP-1 cells which have phagocytosed *M. tuberculosis* strains H37-Rv and H37-Ra (National Collection of Type Cultures, Colindale, UK) [16]. Stimulation of THP-1 cells by purified 65-kD hsp resulted in accumulation of TNF mRNA in similar amounts to that following stimulation by high concentrations of *Escherichia coli* LPS (10 μg/10^7 cells) (Fig. 1a). Such accumulation was not seen following stimulation by low concentrations of *E. coli* LPS (< 1 ng/10^7 cells). As 65-kD hsp is heal labile but LPS is not, TNF gene expression at 90 min was compared in experiments where the soluble stimuli were either preheated at 65°C for 1 h or not heat treated. Such heat treatment will cause almost complete proteolysis. TNF mRNA accumulation after hsp stimulation was found to be heat-sensitive in contrast to that following stimulation by LPS (Fig. 1b). Furthermore, there was a dose-dependent effect of 65-kD hsp on TNF gene expression such that even 0·01 μg/ml of protein resulted in TNF mRNA accumulation (Fig. 1c). This quantity of 65-kD hsp contained less than 1 pg/ml of LPS. Following the demonstration that 65-kD hsp was a stimulus to TNF gene expression, kinetic experiments on gene expression and secretion of TNF were performed. These revealed rapid, transient TNF mRNA accumulation between 1 and 2 h after stimulation of THP-1 cells by 65-kD hsp which was followed by secretion of the cytokine into culture medium, reaching maximal concentrations at 4 h (Fig. 1d).

IL-6 and IL-8 gene expression and secretion

Kinetic studies on IL-6 and IL-8 accumulation following stimulation of THP-1 cells by Detoxigel-purified 65-kD hsp showed that these cytokines are also detectable in culture medium. IL-6 was present 4 h post-stimulation, rose to peak values by 8 h and was still present at 24 h (Fig. 2a). IL-6 gene expression is not detected in THP-1 cells by Northern analysis at these low levels of secreted IL-6. IL-8 secretion began within 1 h of stimulation by 65-kD hsp but did not reach maximal values until 24 h (Fig. 2b). However, there was no change in IL-8 gene expression in the THP-1 cells which constitutively express this mRNA species.

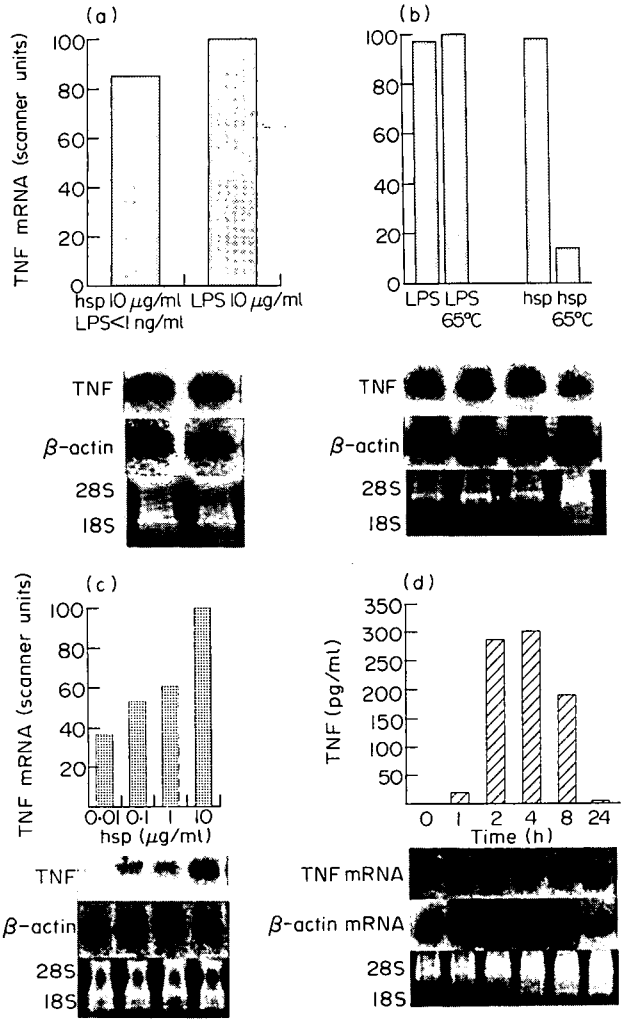

Fig. 1. THP-1 cells accumulate tumour necrosis factor (TNF) mRNA after stimulation with Detoxigel-purified mycobacterial 65-kD heat shock protein (hsp) (lipopolysaccharide (LPS) concentration < 1 ng/ml). (a) Maximal TNF mRNA accumulation (90 min) due to hsp (10 μg/ml) is similar to that occurring following stimulation with LPS (10 μg/ml). (b) TNF mRNA accumulation due to hsp but not to LPS is sensitive to heating to 65°C for 1 h, further indicating that the observed effect of hsp is not a consequence of contamination by LPS. (c) TNF mRNA accumulation in THP-1 cells stimulated by increasing concentrations of 65-kD hsp. At 0·01 μg/ml of protein (< 1 pg/ml LPS) there is detectable TNF gene expression. (d) Kinetics of TNF mRNA accumulation and secretion of this cytokine (▨) by 10^7 THP-1 cells show an early, transient peak of specific RNA followed by detection of TNF in the cell supernatant. Equivalent loading of mRNA is seen from the appearance of 18S and 28S rRNA bands and TNF mRNA units were adjusted for total RNA based upon relative intensity of β-actin mRNA bands. Results (a, b, c and d) are each representative of at least three independent experiments.

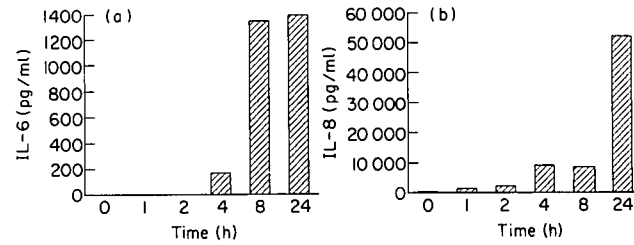

Fig. 2. Kinetics of secretion of (a) IL-6 and (b) IL-8 by THP-1 cells stimulated with Detoxigel-purified mycobacterial 65-kD heat shock protein (hsp). 65-kD hsp was added to medium containing 10^7 cells to a final concentration of 10 μg/ml incubated in six separate PTFE vials. IL-6 and IL-8 released into the medium at the times indicated were assayed as described in text. Results are representative of at least three independent experiments.

DISCUSSION

This study has shown that stimulation of the human monocytic cell line THP-1 by mycobacterial 65-kD hsp results in gene expression and secretion of TNF. Thus, monocytes as well as T lymphocytes may be involved in cellular immune responses to 65-kD hsp and this protein activates monocyte functions other than antigen presentation, in particular cytokine gene expression and secretion. This effect was directly due to the 65-kD hsp and not to contaminating LPS from the recombinant protein (Fig. 1a–c). First, LPS was extracted from samples by a sensitive column purification method. Following this procedure it was shown that the 65-kD hsp had remained intact and on direct measurement using an established limulus amoebocyte assay there was less than 1 ng of LPS per 10 μg of 65-kD hsp. The magnitude of TNF mRNA accumulation in such samples with minimal LPS contamination was very similar to that observed with similar quantities of LPS. In addition, proteolysis induced by heating to 65°C (use of proteolytic enzymes would have been an alternative approach) abolished TNF gene expression due to 65-kD hsp but not that due to heat-stable LPS. The effect of 65-kD hsp on TNF mRNA accumulation was dose dependent and an effect was seen at protein concentrations of 0·01 μg/ml when samples contained less than a total amount of 5 pg LPS (1 pg/ml). Thus, this evidence all indicated that the observed effect of 65-kD hsp on TNF gene expression was not due to LPS.

The kinetics of TNF gene expression and release from THP-1 cells following stimulation by 65-kD hsp was similar to that previously described following stimulation by both LPS and live virulent mycobacteria [9,16]. However, this does not imply that the 65-kD hsp has LPS-like properties but rather that the monocyte pattern of TNF release is relatively similar independent of stimulus. However, TNF release is potentially important in granuloma formation in mycobacterial infection [11]. In addition, TNF is known to have a direct antimycobacterial action *in vitro* [7] and to be involved in elimination of intracellular pathogens *in vivo* [28]. The exact extent to which TNF release due to 65-kD hsp affects mycobacterial replication remains to be elucidated.

Mycobacterial 65-kD hsp directly stimulated early secretion of IL-6 and IL-8 from THP-1 cells. However, the quantities of IL-8 released into tissue culture medium were considerably less (at least two orders of magnitude) than that observed following phagocytosis of virulent *M. tuberculosis* [16]. There may be additional positive feedback of IL-6 and IL-8 secretion via release of autologous TNF. We did not detect altered gene expression of these proinflammatory cytokines. Northern analysis lacked sensitivity to detect alterations in IL-6 mRNA. In contrast, the IL-8 gene was constitutively expressed in THP-1 cells, but increased IL-8 secretion following 65-kD hsp stimulation may be dependent on translational control mechanisms. IL-6, as well as TNF, may be directly involved in host defence to mycobacteria, restricting replication of the organism within macrophages [8]. In addition, both IL-6 and IL-8 modulate T

lymphocyte functions. IL-6 induces progression of T lymphocytes from the G_0 phase to the G_1 phase of the cell cycle [29]. IL-8, principally associated with neutrophil recruitment in human infection [30], is also chemotactic for T lymphocytes *in vitro* and *in vivo* [15]. IL-8 secretion from monocytes stimulated by 65-kD hsp may be involved in recruitment of T cells which may then be followed by T cell proliferation controlled by IL-6. More research is needed to establish such a hypothesis. The fact that relatively low concentrations of these cytokines were secreted following 65-kD hsp stimulation of THP-1 cells suggests that this protein would be only one of many factors involved.

Thus, the monocyte response to 65-kD hsp may be central to the process of granuloma formation. However, a wide spectrum of protein antigens may be involved in the immune response to *M. tuberculosis* [31,32] as well as other cell wall components such as lipoarabinomannan [33]. Furthermore, it is not known exactly when during the course of intracellular infection *in vivo* 65-kD hsp will be expressed by mycobacteria and be able to affect monocyte functions. This, it cannot be proposed that 65-kD hsp is the mycobacterial equivalent of LPS and indeed this seems unlikely. However, interaction between 65-kD hsp and monocytes may be involved in early-onset cytokine-mediated host defence mechanisms to intracellular microorganisms. Since protein chaperonin molecules highly homologous to mycobacterial 65-kD hsp are ubiquitous [34], our next step is to examine THP-1 cells and human monocyte cytokine production in response to other hsp such as groEL from *E. coli*.

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