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Peritoneal macrophages show increased cytokine gene expression following haemorrhagic shock

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SUMMARY

Tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1 and transforming growth factor-β (TGF-β) have been recognized as important mediators of pathophysiological and immunological events associated with shock. Previous studies have indicated that although peritoneal macrophage (PMϕ) antigen presentation was depressed following haemorrhage, the cytokine release capacity in response to lipopolysaccharide (LPS) was not affected *in vitro*. To determine the effect of haemorrhagic shock on PMϕ cytokine mRNA transcription, C3H/HeN male mice were bled to and maintained at a mean arterial blood pressure of 35 mmHg for 60 min, and then adequately resuscitated. PMϕ were isolated at 1 or 24 hr after haemorrhage and were incubated without or with 10 μg LPS/ml for 1 hr. Total RNA was then extracted followed by Northern blot analysis, as well as semi-quantitative reverse transcription and polymerase chain reaction (RT-PCR). The results of Northern blot analysis indicated that haemorrhage markedly increased LPS-induced IL-1β, IL-6, and TNF-α mRNA accumulation in PMϕ at both 1 and 24 hr after haemorrhage and resuscitation. Furthermore, competitive RT-PCR demonstrated that mRNA of IL-1β, IL-6, TNF-α, as well as TGF-β, was increased in PMϕ obtained 1 hr after haemorrhage either with or without LPS stimulation. The data from Northern blot analysis and semi-quantitative RT-PCR also revealed that LPS enhanced the effect of haemorrhage on PMϕ cytokine gene expression. Thus, following haemorrhage, PMϕ showed elevated cytokine mRNA accumulation which was not followed by an increased ability to release cytokines in response to LPS *in vitro*. These results, therefore, suggest that different mechanisms regulate gene expression and subsequent cytokine secretion by PMϕ following haemorrhage.

INTRODUCTION

Tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1 and transforming growth factor-β (TGF-β) are potent inflammatory mediators which produce a broad spectrum of activities in immunological and pathophysiological events.[1,2] It has been reported that the increased serum TNF level in patients with meningococcal sepsis is associated with poor outcome[3] and that the increased serum IL-6 level during sepsis predicts the severity of the illness.[4] Recent studies from our laboratory indicated that simple haemorrhage without significant tissue trauma induced profound immunosuppression which was associated with increased circulating levels of TNF-α, IL-6 and TGF-β.[5,6] Studies have also shown that following haemorrhage, the antigen presentation (AP) capacity of peritoneal macrophages (PMϕ) was significantly depressed and this was associated with the loss of major histocompatibility complex (MHC) class II (Ia) antigens and decreased antigen catabolism.[7,8] Furthermore, studies have showed that ibuprofen administered *in vivo* following haemorrhage markedly reduced plasma prostaglandin E_2 (PGE_2) levels and increased PMϕ AP, as well as Ia expression. These findings suggest that prostaglandins may be involved in the suppression of PMϕ functions following haemorrhage.[9] Nevertheless, despite the above alterations in PMϕ function after haemorrhage, PMϕ cytokine release capacity was not affected *in vitro*.[10,11] Since the mechanism of cytokine regulation in PMϕ after haemorrhage is still unclear, the present study was carried out to determine whether PMϕ cytokine gene expression is affected by haemorrhage.

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MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) was purchased from Difco (Detroit,

Table 1. Predicted PCR products (bp) (Clontech Labs., Inc., Palo Alto, CA)

IL-1β		IL-6		TNF-α		TGF-β	
Target	Mimic	Target	Mimic	Target	Mimic	Target	Mimic
563	420	638	450	692	500	525	390

MI). All reagents for reverse transcription (RT) and polymerase chain reaction (PCR) were obtained from Perkin-Elmer (Roche Molecular Systems Inc., Branchburg, NJ). Clontech Laboratories Inc. (Palo Alto, CA) was the source of synthetic 30-mer oligonucleotide probes for mouse β-actin, IL-1β, IL-6 and TNF-α, as well as cytokine primers and PCR MIMICS (competitive internal standard). The competitive DNA MIMICS are non-homologous DNA fragments which have been added with the primer templates to compete with the target DNA for the same primers. The PCR products amplified from DNA competitive DNA mimic and target DNA can be distinguished by their size (Table 1).

Animals

Inbred C3H/HeN male mice (Charles River Labs., Portage, MI), 6–8 weeks old (weighing 20–25 g), were fasted 18 hr before the experiment, but allowed water *ad libitum*. The studies performed here were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the All-University Committee on Animal Use and Care.

Haemorrhage model

Haemorrhage was induced by the method described in detail by Stephan *et al.*[12] In brief, following light anaesthesia, both femoral arteries were cannulated under aseptic conditions. One catheter was used for constant blood pressure monitoring and and another for haemorrhage and resuscitation. The animals were bled to a mean blood pressure of 35 mmHg, maintained for 60 min at that blood pressure, and then adequately resuscitated with their own shed blood and Ringer's lactate solution (two times the volume of the shed blood). There was no mortality with this model. Sham animals underwent the same anaesthetic and surgical procedures, which included ligation of both femoral arteries, but haemorrhage was not produced.

Preparation of peritoneal macrophages

Resting PMϕ were harvested by peritoneal lavage, using 10 ml of ice cold Click's medium (Irvine Sci., Santa Ana, CA) as previously described by Ayala *et al.*[7] Cells were washed once with Click's medium (280 *g*, 15 min, 4°); the pellet was dispersed and viability determined by trypan blue exclusion. PMϕ were adjusted at a final concentration of 1×10^6/ml in Click's medium. For Northern blot, 8×10^6 PMϕ were plated in a 60-mm Petri dish, and for RT-PCR 3×10^6 PMϕ in a 30-mm Petri dish. After 2 hr at 37° in a 5% CO_2 atmosphere, non-adherent and non-viable cells were removed by three repeated washings. No difference in cell yields and viability was observed between normal, sham-operated and haemorrhaged animals.

RNA extraction and Northern blot analysis

Total RNA was extracted from PMϕ with or without LPS stimulation for different intervals, according to experimental design. A modification of the methods of Chirgwin *et al.*[13] and Jonas *et al.*[14] was used. Briefly, macrophage monolayers were lysed with a solution of 25 mM Tris containing 4·2 M guanidine isothiocyanate, 0·5% Sarkosyl and 0·1 M 2-mercaptoethanol, followed by extraction with phenol/chloroform and chloroform/isoamyl alcohol. The extracted RNA was precipitated in alcohol and the pellet was dissolved in TE buffer (Tris 10 mM/EDTA 0·1 mM). RNA concentration and purification were determined by measuring the absorbance at 260 and 280 nm. For Northern blot analysis, total RNA (10 μg/lane) was fractionated in 1% agarose/2·2 M formaldehyde gel. RNA was transferred to nitrocellulose (Schleicher & Schuell Inc., Keene, NH) and fixed by ultraviolet light (UV Stratalinker, Stratagene, La Jolla, CA). Blots were prehybridized and hybridized with probes for TNF-α, IL-1β, IL-6 and β-actin mRNA sequentially, at 57° in 6 × sodium chloride/sodium citrate buffer (SSC), 1 × Denhardt's, 100 μg/ml transfer RNA, 0·05% sodium pyrophosphate and 50 μg/ml polyadenylic acid. Blots were washed with 2 × SSC/0·05% sodium pyrophosphate at 57°. Autoradiography was carried out using Kodak X-OMat film and an intensifying screen at −70°. The band intensities of the autoradiograms were evaluated by the JAVA Image Analysis System (Jandel Scientific, San Rafael, CA) and normalized by intensity of β-actin bands.

Determination of the kinetics of LPS-induced cytokine gene expression

PMϕ from normal mice were obtained as described above. Adherent PMϕ were stimulated without or with LPS (10 μg/ml) for 30 min, 1 hr, 2 hr and 4 hr. Total RNA was isolated following the above time-points, and Northern blot analysis carried out as described above. Based upon the results of the time–course (Fig. 1), we chose the 1 hr exposure to LPS as the time-point for subsequent experiments.

Measurement of cytokine gene expression after haemorrhage by Northern blot analysis

Mice were killed at 1 hr or 24 hr following haemorrhage and resuscitation or sham operation. Adherent PMϕ were stimulated without or with LPS (10 μg/ml) for 1 hr at 37° in 5% CO_2. Total RNA was isolated at the end of LPS stimulation, and Northern blot analyses for TNF-α, IL-1β, IL-6 and β-actin gene expression were performed according to the methods described above.

Measurement of cytokine gene expression after haemorrhage by semi-quantitative RT-PCR

PMϕ were harvested at 1 hr after haemorrhage or sham operation. Two micrograms of RNA from PMϕ stimulated without or with LPS (10 μg/ml) for 1 hr was reverse transcribed in a 20 μl reaction volume containing 1 × RT buffer (50 mM KCl, 10 mM Tris–HCl), 5 mM $MgCl_2$, 1 mM dNTP, 20 U RNase inhibitor, 2·5 mM oligo d(T)16 primer and 50 U RT. The RT reaction was incubated at 42° for 1 hr, followed by heating at 99° for 5 min. Five per cent portions of cDNA were amplified with 0·15 μM each of 3′ and 5′ primers, specific for TNF-α, IL-1α, IL-1β, IL-6 and TGF-β, in the presence of a certain consistent competitive internal standard (PCR MIMIC) in

25 μl of PCR mixture containing 1 × buffer (50 mM KCl, 10 mM Tris–HCl), 2 mM $MgCl_2$, 0·2 mM dNTP and 0·7 U AmpliTaq DNA polymerase. PCR was carried out in a Perkin-Elmer/Cetus thermal cycler 9600. PCR mixtures were heated at 95° for 2 min followed by 35 cycles, each consisting of 45 seconds at 94°, 45 seconds at 60° and 2 min at 72° incubations.

The proper concentration of PCR MIMIC for each cytokine was established in a preliminary study (data not shown) so that target gene transcripts could be analysed accurately and quantitatively. In brief, the higher the level of target mRNA, the more competitive internal standard was needed. For the samples which were stimulated with LPS, 10^{-1} attomole PCR MIMIC of all four cytokines was added to the PCR reaction. In comparison, for the samples which were not stimulated with LPS, 10^{-5} attomole MIMIC was chosen for quantification of TNF-α and TGF-β gene expression, 10^{-3} attomole for IL-1β and 10^{-2} attomole for IL-6.

Detection of PCR products

Seven microlitres of the RT-PCR mixture (30% of the portion) was electrophoresed in 1·8% tris/borate/ethylenediaminetetraacetic acid (TBE)–agarose gel containing 0·5 μg/ml ethidium bromide. The gel was photographed with Polaroid film. The amount of PCR products generated by the target and the competitive internal standard were measured by Mocha Image System (Jandel Scientific); the ratio of target to internal standard was calculated. The difference in the ratio between haemorrhage and sham represented the amount of change in mRNA level.

The results of typical experiments, repeated no less than three times, are presented here.

RESULTS

Kinetics of LPS-induced cytokine gene expression

Northern blot analysis showed that the induction of TNF-α mRNA was very rapid, reaching a peak at 0·5 hr after LPS stimulation and declining thereafter to baseline at 2 hr following LPS addition (Fig. 1). In comparison to TNF-α, IL-1β and IL-6 gene expression increased later, starting at 1 hr and continuing to increase throughout the entire time–course of the experiment.

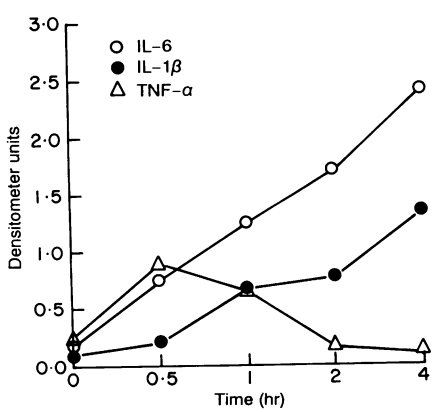

Figure 1. Kinetics of cytokine mRNA transcripts after LPS stimulation. PMϕ from normal C3H/HeN mice were stimulated with LPS 10 μg/ml for 0 min, 0·5 hr, 1 hr, 2 hr and 4 hr. Total RNA was isolated and subjected to Northern blot analysis for TNF-α, IL-1β and IL-6. The graph shows the density of the blots measured by JAVA Image System and normalized by β-actin.

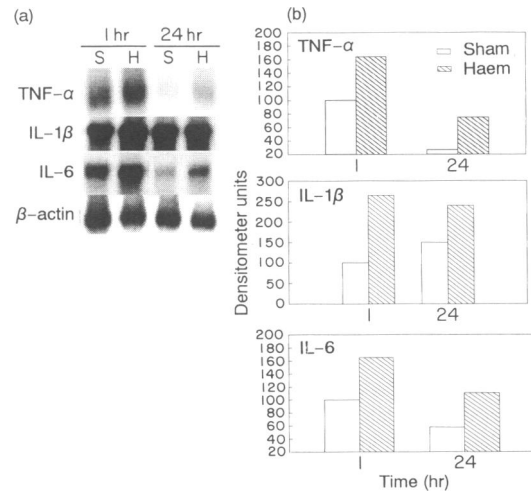

Figure 2. Northern blot analysis of cytokine mRNA accumulation after haemorrhage. PMϕ were obtained at 1 and 24 hr following haemorrhage and stimulated with LPS (10 μg/ml) for 1 hr. Total RNA was extracted, blotted and sequentially probed for TNF-α, IL-1β, IL-6 and β-actin. (a) Northern blot for TNF-α, IL-1β, IL-6 and β-actin. (b) Histograms show the densitometer reading from the corresponding blot which was normalized by β-actin. The results shown are from a representative experiment. Similar results were obtained from three repeated experiments.

Cytokine gene expression by Northern blot analysis after haemorrhage

Figure 2 shows that LPS-induced TNF-α mRNA was increased by approximately 64% at 1 hr and 48% at 24 hr after haemorrhage, respectively. Similarly, IL-1β and IL-6 mRNA accumulation in response to LPS were enhanced at both 1 hr and 24 hr after haemorrhage. Cytokine genes in PMϕ without LPS stimulation were not detectable by Northern blot analysis, either from haemorrhage or sham mice (data not shown).

Cytokine gene expression after haemorrhage by semi-quantitative RT-PCR

The results show that haemorrhagic shock enhanced mRNA accumulation of TNF-α, IL-1β, IL-6 and TGF-β in PMϕ stimulated with LPS (Fig. 3). Of further interest was the finding that PMϕ without LPS stimulation exhibited differential cytokine mRNA expression (Fig. 4).

Following haemorrhage, mRNA of IL-1β, TNF-α and TGF-β, but not IL-6, were increased in PMϕ without LPS stimulation. Interestingly, while cytokine gene expression was augmented by LPS, the differences in the mRNA level between the various cytokines were reduced. Unlike IL-1β, mRNA for IL-1α was not detectable in PMϕ with or without LPS

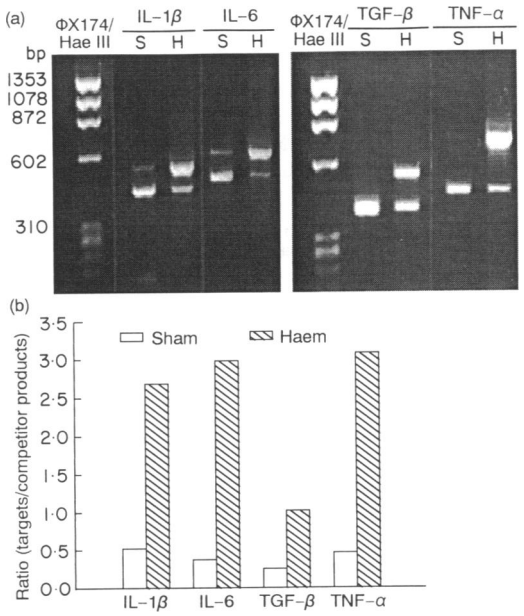

Figure 3. Semi-quantitative RT-PCR to examine the level of cytokine gene transcripts in PMϕ with LPS stimulation post-haemorrhage. PMϕ harvested at 1 hr after haemorrhage and sham were co-incubated with LPS 10 μg/ml for 1 hr. Total RNA was extracted and cDNA was synthesized as described in the Materials and Methods. (a) A constant amount of PCR MIMIC (10^{-1} attomole) for IL-1β, IL-6, TNF-α and TGF-β was co-amplified with 4% portions of cDNA and then 30% of PCR products were resolved on a 1·8% EtBr–agarose gel. (b) Histogram representing the result in (a) after the Polaroid films were analysed by Mocha Image System and the ratio of target to competitive internal standard calculated. The results shown are from a representative experiment. Similar results were obtained from three repeated experiments.

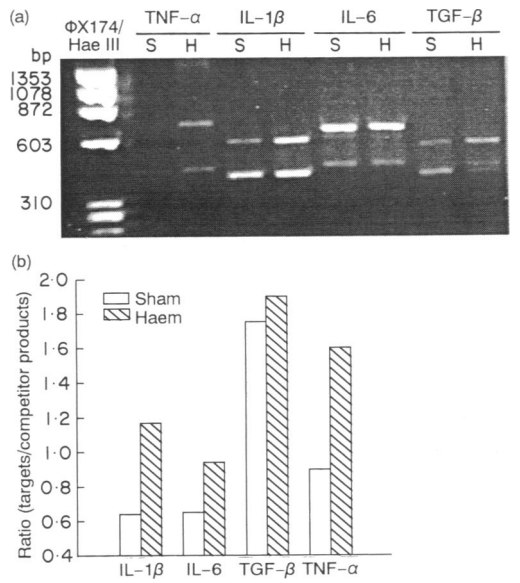

Figure 4. Competitive RT-PCR analysis of cytokine mRNA alternation in PMϕ after haemorrhage and sham. Total RNA was extracted from PMϕ obtained 1 hr following haemorrhage and sham. Four per cent portions of cDNA were amplified with primers for IL-1β, IL-6, TNF-α and TGF-β in the presence of different amounts of PCR MIMIC (10^{-3} attomole for IL-1β, 10^{-2} attomole for IL-6 and 10^{-5} for TGF-β as well as TNF-α). (a) Thirty per cent of PCR products were analysed by 1·8% EtBr–agarose gel electrophoresis. (b) Histogram showing the result in (a) after the Polaroid film was analysed by Mocha Image System and the ratio of target to competitive internal standard calculated. The results shown are from a representative experiment. Similar results were obtained from three repeated experiments.

stimulation and, therefore, was too low to be quantified by RT-PCR in this study (data not shown).

DISCUSSION

Cytokines are potent inflammatory mediators of shock and have been investigated intensively over the past few years.[5,6,11,15–17] TNF-α has been recognized as a primary mediator which initiates the elaboration of a cascade of other mediators.[1,18,19] The pleiotropic role of IL-1 and IL-6 are also well established in inflammatory, metabolic, haematopoietic and immunological processes.[1,20,21] Furthermore, TGF-β is known to affect cells involved in immunity and inflammation.[1,2,22] Studies have demonstrated that haemorrhagic shock without significant trauma produces severe immunosuppression which is associated with increased cytokine release *in vivo* and *in vitro*.[5,6,11,15] However, previous investigations from our laboratory indicated that while PMϕ showed depressed antigen presentation function following haemorrhage, the cytokine productive capacity was not enhanced.[7,10] This study was therefore conducted to determine the effect of haemorrhage on PMϕ at the transcriptional level of several cytokines.

The time–course of LPS-induced cytokine mRNA accumulation in resting PMϕ from normal mice indicated that TNF-α gene expression was rapidly increased in response to LPS, reaching a peak at 30 min and rapidly disappearing thereafter. IL-1β and IL-6 gene expression lagged behind TNF-α, starting to increase at 1 hr and reaching a maximum at 4 hr of LPS stimulation. These observations are comparable to the results of others, which indicate that cytokine transcription occurs in a sequential fashion.[23,24] In contrast, non-elicited PMϕ from CBA/J mice failed to synthesize TNF mRNA or bioactive material in response to LPS stimulation, our results dealing with the kinetics of resting PMϕ from C3H/HeN mice are similar to those obtained from elicited PMϕ of CBA/J mice.[25] This implies that differences in the strain of mouse utilized might account for these different results.

Our preliminary studies using Northern blot analysis revealed that haemorrhagic shock significantly up-regulated mRNA levels of IL-1β, IL-6 and TNF-α only in LPS-stimulated PMϕ. The effect of haemorrhage on cytokine gene expression was seen as early as 1 hr but was still present 24 hr after haemorrhage. However, it was observed that without LPS stimulation, cytokine mRNA transcripts in PMϕ were not detectable by Northern blot analysis on the cells either from sham or haemorrhaged mice. The inability to detect cytokine mRNA of PMϕ in the absence of LPS stimulation might be due to the fact that Northern blot analysis is not sensitive enough to determine the minute quantity of mRNA in PMϕ. Therefore, in order to gain further information concerning the effect of haemorrhage on PMϕ mRNA transcripts, semi-quantitative RT-PCR was employed to examine the level of mRNA transcripts of IL-1α, IL-1β, IL-6, TNF-α and TGF-β. Using RT-PCR analysis, it was demonstrated that in PMϕ stimulated

with LPS, all the above cytokine mRNA accumulations, except IL-1α, were significantly augmented after haemorrhage. These results serve to confirm the initial findings made with the Northern blot analysis. However, semi-quantitative RT-PCR analysis revealed that there was differential regulation of mRNA transcripts among cytokines in PMϕ without LPS stimulation. It was also noted that, at 1 hr after haemorrhage, mRNA accumulation of IL-1β, TNF-α and TGF-β were markedly elevated, but IL-6 mRNA was not significantly different between haemorrhage and sham. One could speculate that the tissue injury and anaesthesia required to catheterize these animals could be sufficient to induce significant levels of IL-6 prior to haemorrhage. In this regard, we have previously reported that trauma alone (without accompanying blood loss) can induce IL-6 release.[5] However, in the absence of naive (i.e. without sham operation) control animals the mechanism of this phenomenon remains unknown. Furthermore, our results demonstrate that LPS, a potent stimulant of immune cells, acts to enhance the cytokines' mRNA expression in PMϕ by 10^4 times, according to the known amount of competitive internal standards added into the PCR reactions. Interestingly, LPS stimulation seemed to reduce the degree of differences in mRNA expression while up-regulating cytokine mRNA transcripts. No detectable IL-1α mRNA was found in PMϕ without LPS stimulation following haemorrhage or sham. In spite of LPS stimulation, IL-1α mRNA was too low to be quantified by this method. It is known that IL-1α and IL-1β are products of distinct genes which are often differentially expressed depending on the type of cells, the state of proliferation, tissue distribution or even the time in culture.[26] The present experiment demonstrates that PMϕ from C3H/HeN mice express mainly IL-1β mRNA and little, if any, IL-1α mRNA.

The finding that cytokine gene expression in ^{D}Mϕ is enhanced following haemorrhage may appear at odds with our previous results, which indicated that PMϕ did not show increased release of IL-1, IL-6 and TNF-α as measured by bioassay.[7,10] However, in this regard, recent studies have reported that increased cytokine gene expression does not always manifest itself as an increase in secreted cytokine.[27,28] In other words, the transcription of genes alone does not always correspond qualitatively or quantitatively to translation.[28] A second signal or a more intense stimulation may be required to induce secretion of the cytokine. The precise nature of the discrepancy between transcription and translation/secretion of these cytokines by PMϕ following haemorrhage remains to be determined.

Thus, following haemorrhage, PMϕ show differentially elevated cytokine mRNA accumulation which, however, is not followed by an increased ability to release cytokines in response to LPS *in vitro*. These results, therefore, suggest that different mechanisms regulate gene expression and subsequent cytokine secretion by PMϕ following haemorrhage.

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