

## EFFECTS OF ENDOTOXIN ON SERUM CHEMOKINES IN MAN

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### Abstract

*Aim:* Endotoxin is known to be a primary initiator of sepsis and septic shock. Migration of immunocompetent cells due to chemotactic attraction plays a central role in the initiation of the immune response. Two major groups of chemokines can be distinguished: C-x-C chemokines like Interleukin-8 attract mainly neutrophils, C-C chemokines (e.g. RANTES) attract monocytes and T-cells. The aim of this study was to get further insight into chemokine profiles after a single endotoxin bolus in man.

*Materials and Methods:* We investigated the effect of systemically administered endotoxin (4ng/kg BW i.v.) in 8 healthy volunteers.

Clinical data (heart rate, mean arterial pressure, temperature), serum levels of IL-8, and RANTES, as well as white blood cell count were obtained before and hourly for five hours after endotoxin administration.

*Results:* Heart rate and MAP showed significant changes ( $p < 0.05$ ) after 2-3 hours. All volunteers presented with low-grade fever after 2 hours. WBC was elevated 43% and 63% after 4 and 5 hours, respectively. Both chemokines were significantly different from baseline two hours after endotoxin challenge: While IL-8 was significantly increased RANTES serum levels were diminished.

*Conclusion:* From our data we conclude that this endotoxin model was effective to mimic the clinical appearance of sepsis. Chemokines like IL-8 and RANTES are integrated in the early immune response to endotoxin challenge in man.

*Key words:* Endotoxin; Volunteer Study; Chemokines; Sepsis; IL-8; RANTES

### INTRODUCTION

Movement of leukocytes from the vasculature through the vessel wall and into tissue to the site of inflammation is a complex multistep event. Chemotaxis of leukocytes represents a central mechanism in the inflammatory immune response to microbial products and chemokines appear to play a central role in leukocyte recruitment and activation.

Based upon the first two cysteine residues in their sequence, the chemokines have been divided into two distinct families, the C-x-C (alpha) and the C-C (beta) family. [11, 19]

In addition, the functional activities of these family members also have distinct properties. The members of the C-x-C family like IL-8 or ENA-78 are predominantly chemotactic for neutrophils, whereas C-C family members like RANTES are chemotactic for mononuclear leukocytes, monocytes and lymphocytes. [13]

Due to the diversity of chemokines and their function in diseases it is important to understand their kinetics and their functional role in modulating the immune response including the induction and maintenance of chemotaxis of leukocytes.

The purpose of this study was to investigate the changes in levels of chemokines like IL-8 and RANTES in the early inflammation response in human endotoxemia.

### MATERIALS AND METHODS

#### SUBJECTS

Eight healthy volunteers (4 men and 4 women), with a mean age of  $21.7 \pm 0.8$  years were studied. All subjects had a negative medical history and physical examination, and normal results on blood and urine analyses, electrocardiography and transthoracic echocardiography. The study was approved by the institutional review board, and written informed consent was obtained from each volunteer before enrollment in the study.

Subjects had an 18-ga peripheral intravenous catheter inserted, through which 0.9% saline 2ml/kg/h was infused throughout the study period. Additional fluid boluses of 500ml/h 0.9% saline were given as needed to maintain intravascular volume. Invasive blood pressure was monitored throughout the study, either by a 20-ga radial or femoral arterial catheter. In addition, cardiac output was measured in four of the volunteers through a thermodilution catheter placed in the right internal jugular vein.

Body temperature and hemodynamics, including mean arterial pressure (MAP), and heart rate (HR), were monitored at fifteen minute intervals from the time of catheter insertion. In addition, cardiac index (CI), systemic vascular resistance (SVR), and pulmonary artery occlusion pressures were measured in volunteers with pulmonary arterial catheters. Differential leukocyte and hemoglobin counts, were obtained at baseline, and hourly for five hours following endotoxin bolus. Blood for the cytokine assays, as well as

for RNA studies, was drawn into heparinized tubes from the intravenous catheter before and hourly for 5 hours after the endotoxin injection. Plasma was collected immediately and frozen at  $-70^{\circ}\text{C}$  until assayed. PBMC were isolated over Ficoll Hypaque cushion. The harvested cells were lysed and frozen until further analysed. After completion of the study, all volunteers were monitored for a further three hours until hemodynamics approached baseline values.

After baseline hemodynamic measurements, all volunteers received a bolus of U.S. Reference *E coli* endotoxin 4ng/kg bodyweight (Lot EC-5, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) with a specific activity of 10 units per nanogram.

#### CHEMOKINE ASSAYS

IL-8 and RANTES were detected in plasma with a sandwich ELISA kit from R&D (R&D Systems Inc., Minneapolis, MN, USA).

#### IL-8 MESSENGER RNA ASSAY

Messenger RNA of IL-8 was measured from PBMC, isolated via Ficoll separation. Total RNA was extracted by acid phenol extraction as described by Chomczynski et al. [5] Quantitative RT-PCR (Clontech Laboratories Inc., Palo Alto, CA, USA) in terms of a competitive RT-PCR with internal standard for IL-8 (PCR MIM-ICs, Clontech Laboratories Inc., Palo Alto, CA, USA) was used according to the manufacturers protocol.  $\beta$ -actin was used as a positive control. 10 $\mu\text{g}$  total RNA were used for each sample. Quantification of the PCR products were performed with radioactive labeled dNTP, which was added in the RT-PCR reaction. After gel analysis the gel was dried, an autoradiograph was generated and scanned in a densitometer. According to the manufacturers protocol the amount of target RNA (attomoles (a)) was calculated with a linear regression analysis when the ratio of target to mimic was 1.

#### STATISTICAL ANALYSIS

Data are presented as means  $\pm$  standard errors of the means (SEM). Differences in the experimental means were considered significant at  $p < 0.05$  as determined by Student's paired t-tests and Bonferroni correction for multiple comparisons. For correlation analysis the Pearson correlation coefficient ( $r$ ) was used.

## RESULTS

### PHYSIOLOGICAL CHANGES

All subjects experienced flu-like symptoms including headaches, rigors, muscle pains, stomach ache, and nausea beginning approximately one hour after the endotoxin bolus and lasting for about one hour. In accordance with other investigators all volunteers developed low-grade fever after three hours ( $38.0 \pm 0.1^{\circ}\text{C}$ ;  $p < 0.01$ ). Hemodynamic changes (Table 1) were significantly different from baseline after two to three hours. Tachycardia, as well as a decrease in the MAP, was seen after three hours ( $92 \pm 3$  beats/min,  $80.5 \pm 2.8$  mmHg, respectively;  $p < 0.01$ ). The central venous pressure (CVP) and the SVR decreased after endotoxin administration ( $7.1 \pm 1.4$  vs  $1.3 \pm 0.5$  cm H<sub>2</sub>O;  $1517 \pm 59$  vs  $907 \pm 59$  dynes/cm<sup>5</sup>, respectively). CI increased from 3.6 l/min/m<sup>2</sup> to 5.7 l/min/m<sup>2</sup> after endotoxin injection. The white blood cell count increased gradually during the observation period (pre-endotoxin  $6.8 \pm 0.8$  10<sup>3</sup>/cmm vs five hours post-endotoxin  $10.1 \pm 1.0$  10<sup>3</sup>/cmm).

The differential count showed a shift to immature neutrophils (bands) after two hours ( $30.7 \pm 2.8\%$ ;  $p < 0.01$ ), with an accompanying decrease of lymphocytes ( $9.7 \pm 1.5\%$ ;  $p < 0.01$ ) and monocytes after three hours ( $0.6 \pm 0.2\%$ ;  $p < 0.01$ ) (Fig. 1).

#### IL-8

IL-8 serum levels were significantly elevated after 2 hours (296 pg/ml) and declined thereafter. Interestingly the course of IL-8 in serum and of the immature neutrophils were almost identical and had a significant correlation of 0.77 ( $p < 0.05$ ).

RNA studies in circulating blood mononuclear cells for IL-8 with RT-PCR revealed that IL-8 mRNA is also increases after 2-3 hours (0h: 15.3 a; 3h: 268 a) (Fig. 2).

#### RANTES

RANTES as a C-C chemokine family member was significantly decreased 2 hours (936 pg/ml) after endotoxin challenge. We could show a significant correlation between Rantes and peripheral blood monocytes ( $r = 0.96$ ;  $p < 0.005$ ) (Fig. 3).

Table 1. Hemodynamic changes (HR – heart rate, MAP – mean arterial pressure, CVP – central venous pressure, CI – cardiac index, SVR – systemic vascular resistance) during the experimental setting, measured before and hourly after the endotoxin administration for five hours. \*\* $p < 0.01$

Hemodynamic variable	baseline	1h	2h	3h	4h	5h
HR (beats/min)	69 $\pm$ 3	75 $\pm$ 4	86 $\pm$ 5**	92 $\pm$ 3**	94 $\pm$ 4**	88 $\pm$ 3**
MAP (mmHg)	98 $\pm$ 3	95 $\pm$ 3	86 $\pm$ 4	81 $\pm$ 3**	80 $\pm$ 3**	83 $\pm$ 4**
CVP (cm H <sub>2</sub> O)	7.1 $\pm$ 1.4	3.6 $\pm$ 1.1	2.3 $\pm$ 0.6**	1.3 $\pm$ 0.5**	1.6 $\pm$ 0.6**	1.6 $\pm$ 0.5**
CI (l/min/m <sup>2</sup> )	3.6 $\pm$ 0.2	3.8 $\pm$ 0.2	5.7 $\pm$ 0.5**	6.0 $\pm$ 0.3**	5.6 $\pm$ 0.3**	5.4 $\pm$ 0.3**
SVR (dynes/cm <sup>5</sup> )	1517 $\pm$ 59	1480 $\pm$ 87	909 $\pm$ 59**	782 $\pm$ 33**	846 $\pm$ 55**	861 $\pm$ 35**

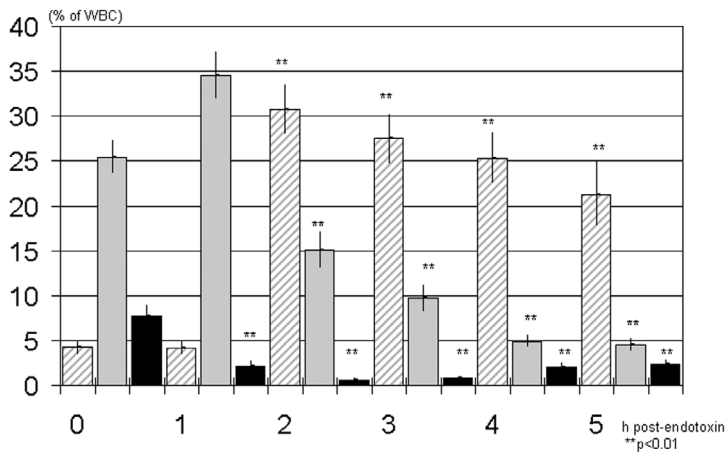


Fig. 1. Immature granulocytes (stripes), lymphocytes (gray) and monocytes (black) before and five hours consecutively after the endotoxin challenge as % of the total white blood cell count. (\*\* $p < 0.01$  vs baseline values timepoint 0)

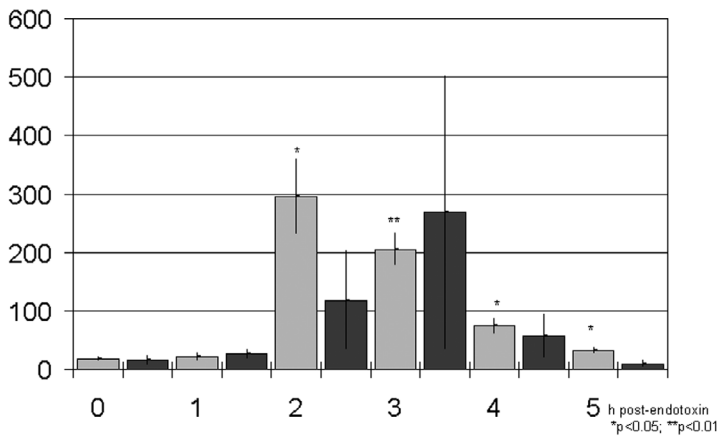


Fig. 2. IL-8 protein (gray; pg/ml) and IL-8 mRNA (black; a) levels of the volunteers before and for five hours after endotoxin challenge. (\* $p < 0.05$ ; \*\* $p < 0.01$ )

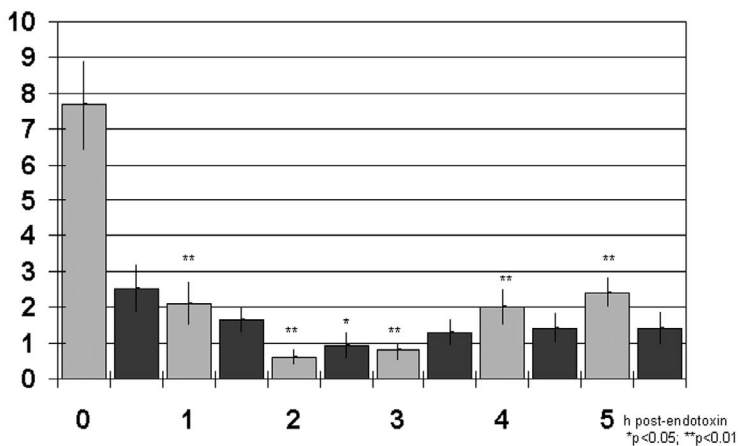


Fig. 3. Levels of monocytes (gray; % of WBC) and rantes (black; pg/ml) at baseline and for five hours post-endotoxin. (\* $p < 0.05$ ; \*\* $p < 0.01$ )

## DISCUSSION

The importance of chemotactic cytokines in the acute immunoresponse to endotoxin is established, but distinct information about regulatory patterns and kinetics remain yet unclear.

Previous studies in human endotoxemia models have focussed on a variety of pro- and antiinflammatory cytokines, such as TNF- $\alpha$ , IL-1, or lymphokines [9, 16, 18, 28].

To reveal further insights in the role of chemokines in the early phase of immunoactivation in men to a endotoxin bolus we investigated the kinetics of members of the two chemokine families C-C (RANTES) and C-x-C

(IL-8) after endotoxin challenge in human volunteers.

As demonstrated in previous human endotoxin studies that used the same model we saw a hyperdynamic circulatory and cellular response as seen in acute sepsis [3, 9, 16, 18].

After low-dose endotoxin administration [15] all volunteers developed symptoms that are associated with septic responses. Our data demonstrate that endotoxin administration resulted in a hyperdynamic cardiovascular response. Two hours after the injection the CI and HR increased, while the SVR and the MAP fell. Fever occurred after three hours, and changes in the blood cell composition (granulocytosis, lymphopenia and monocytopenia) were apparent already one

hour after the endotoxin bolus. All these responses are qualitatively similar to those associated with sepsis and septic shock. [3] Our data demonstrate that a bolus of 4ng/kg endotoxin was sufficient to induce sepsis-like symptoms and reveals comparable data as did other in-vivo endotoxin studies in animals and humans.

In this study we were able to show that there are distinct patterns of circulating chemokines of both families C-C and C-x-C after low-dose endotoxin administration.

We found increased levels of plasma IL-8 and IL-8 mRNA in PBMC. Changes in plasma levels of IL-8 correlated with the kinetics of immature polymorph nuclear cells (bands) reflecting an immediate emergency recruitment from the bone marrow. IL-8 seems to play a role in the acute phase of endotoxemia, and it may be involved in the attraction of immature cells.

In contrast the C-C chemokine RANTES was decreased in serum and correlated with the reduced monocyte count supporting the hypothesis that RANTES plays a subsidiary role in the acute phase of this setting.

Several studies have shown the systemic appearance of pro- and anti-inflammatory cytokines after intravenous endotoxin administration [9, 16, 18]. In our previous studies we have identified several lymphokines that stimulate a shift towards a Th2 polarized immunoresponse [28]. Recent studies by Mantovani et al. support the hypothesis that chemokines contribute to the polarization towards a Th2 response in endotoxemia and sepsis [14].

These studies indicate that neutrophil chemoattractants (C-x-C chemokines) like IL-8 or ENA-78 are responsible for the recruitment of granulocytes from the circulation through the endothelium into the interstitium [2, 24].

Our previous studies have shown that C-x-C chemokines in plasma of severely injured patients play an important role in the early as well in later phases in the immune response to trauma and inflammation suggesting that these mediators may have an important role in neutrophil recruitment. ENA-78 was already significantly elevated at the site of the accident in trauma victims. [20] IL-8 serum levels correlate with the severity of inflammatory disorder [8]. The striking role of C-x-C chemokines in the development of lung diseases such as idiopathic pulmonary fibrosis and adult respiratory distress syndrome (ARDS) as well as in rheumatic diseases has also been shown [4, 7, 25]. IL-8 can also be found in the circulation of primates and humans during septic shock [8, 22], however, IL-8 given intravenously reduces granulocyte recruitment to inflammatory sites by inhibiting functions that are necessary for transmigration, which are independent of granulocyte rolling at the endothelium [12]. These effects of serum IL-8 may underline the prominent and complex role of this cytokine in inflammatory states.

In-vitro studies with LPS in human whole blood revealed that IL-8 mRNA and protein are induced within 1 to 3 hours after LPS, reach a plateau between 6 to 12 hours, and rise again in a second wave which continued to escalate until the end of the 24 hour observation period [6].

Using the same human endotoxin model Suffredini et al. (1991) found that serum IL-8 is induced 1.5

hours after the endotoxin bolus, reaching a maximum at 2 hours and decline thereafter. Pretreatment with oral ibuprofen but not oral pentoxifylline induced a significantly higher IL-8 serum level [16]. In another human endotoxin study van den Blink showed that p38 mitogen activated protein kinase inhibitor could diminish the LPS induced rises in plasma IL-8 [21]. An anti-human CD 14 monoclonal antibody (IC14) was also able to reduce IL-8 plasma levels in an human endotoxin model [17]. Thus our data are consistent with the changes of IL-8 in plasma and add new aspects of mRNA kinetics in-vivo.

Our studies have shown that the suppression of RANTES, compared to controls, correlates with monocyte counts in peripheral blood.

C-C chemokines like MIP-1 alpha and RANTES were shown to be expressed during endotoxemia [23, 26]. The inhibition of these chemokines in-vivo attenuated leucocyte infiltration into the lungs and blocked the lethality associated with endotoxemia. These cytokines are produced later in the response: In mice RANTES increased 2 hours after LPS treatment whereas other C-C and C-x-C chemokines occurred as early as 0.5 hours after challenge. [10] In another animal model it was shown that in the lung and blood cells, maximum RANTES mRNA levels were achieved after 8 hours [23].

In human atopic subjects it was shown that in contrast to other chemokines RANTES were expressed later and may have more relevance to the later accumulation of T-cells and macrophages [27].

Schall et al. have shown that in addition to inducing chemotaxis, RANTES can act as antigen-independent activator of T-cells in-vitro [1].

Thus it seems that the suppression of RANTES in the early phase of endotoxemia does not exclude its importance in the immunoresponse to endotoxemia but might support previous hypothesis that RANTES play a more dominant role in later phases of the immunoresponse to endotoxin or inflammation.

## CONCLUSION

In accordance to prior studies it seems that in the acute phase of experimental endotoxemia in human volunteers the C-x-C chemokine IL-8 is highly activated, and that the C-C chemokine RANTES plays no major role in the early phase of the acute response. The specific pattern of expression of each individual chemokine is likely to reflect its unique function in the regulation and progression of an inflammatory response. The complex orchestration of the chemokines investigated in the response to endotoxin in this human model highlights the necessity for understanding the timing and localization of chemokine expression and production to describe and predict the clinical course of septic patients and to identify potential targets for therapeutic interventions.

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