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Change in sensitivity to lipopolysaccharide during the differentiation of human monocytes to macrophages in vitro

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Abstract. Mononuclear phagocytes in distinct differentiation stages and cultured under different conditions were tested for their sensitivity towards lipopolysaccharide (LPS), using procoagulant activity (PCA) expression and tumor necrosis factor (TNF) production as indices. The response of mature monocyte-derived macrophages differed from that of freshly isolated monocytes 1) by higher levels of constititive PCA, 2) by responding to approximately 1,000-fold lower concentrations of LPS with PCA and TNF production, and 3) by a faster rise in PCA and TNF production. Due to the high constitutive level of PCA expression, the PCA stimulation index for LPS was low in macrophages when compared with that in monocytes. Thus, during differentiation to macrophages, human monocytes acquire increased sensitivity to LPS (2 orders of magnitude more sensitive than a sensitive turbidimetric *Limulus* amoebocyte lysate assay). This exquisite sensitivity to LPS is expressed regardless of whether LPS is offered in the presence or absence of lipopolysaccharide binding protein-containing serum. This points to as yet uncharacterized pathways of high affinity interaction between LPS and macrophages.
Key words. Monocytes; macrophages; procoagulant activity; tumor necrosis factor (TNF); lipopolysaccharide (LPS); macrophage activation.

In mammalian hosts, lipopolysaccharides (LPS) of Gram-negative bacteria and other bacterial constituents are powerful pyrogens and induce a plethora of events which lead, in its most severe form, to shock, as exemplified by the septic shock due to Gram-negative organisms[1-3]. High concentrations of LPS activate Hageman factor, the complement system, and the kinine system, and lead to polyclonal B cell activation[3,4]. Far lower concentrations are required to stimulate the mononuclear phagocyte system which is therefore more relevant for the understanding of LPS-induced pathology. LPS-exposed macrophages produce de novo a variety of secreted and surface-expressed mediators involved in inflammatory and hemostatic processes[1,3]. LPS-induced macrophage mediators include interleukin-1 (IL-1; also referred to as endogenous pyrogen), tumor necrosis-factor (TNF), IL-6 and IL-8[5-7] which collectively have inflammatory, immunostimulatory and catabolic properties. LPS also induces the surface membrane expression of tissue factor[8] which leads to activation of the extrinsic coagulation pathway, resulting in dramatically enhanced procoagulatory activity upon exposure to LPS. TNF and IL-1 induce PCA not only in mononuclear phagocytes themselves[9] but also in endothelial cells[10,11], thereby also contributing to the procoagulatory/thrombogenic properties of LPS-stimulated mononuclear phagocytes.

LPS is thought to activate monocytes/macrophages by interacting with various types of surface receptors[12]. In recent years, attention has focused on a recognition system composed of an acute-phase plasma protein, lipopolysaccharide-binding protein (LBP) and a phosphatidyl inositol-linked surface membrane protein, CD14[13]. CD14 interacts with a complex formed of LPS and LBP which leads to signal-transduction resulting in activation of nuclear factor-kappa-B (NFκB) and other transcription factors[14]. NFκB is required for activation of several macrophage genes, including the TNF and the tissue factor gene[15,16]. LPS-induced signal transduction mediated by CD14 and other putative LPS receptors is still incompletely understood, as is the way how distinct differentiation and activation stages of mononuclear phagocytes influence the interaction with LPS. Here we studied kinetics and dose-response relationship of LPS triggering, and two effector functions, TNF and PCA induction, throughout the differentiation of monocytes to macrophages. The differentiation of human monocytes to macrophages turned out to be associated with markedly altered responsiveness to LPS, characterized, in particular, by the acquisition of high sensitivity towards LPS.

Materials and methods

Human mononuclear cells (MNC) were isolated by isopycnic centrifugation on ficoll-hypaque in the absence of detectable endotoxin contamination as described[17,18]. Freshly isolated cells to be stimulated by endotoxin were subcultured in microtiter plates in PCA medium. This consisted of RPMI 1640 fortified with L-glutamine (2 mM), non-essential amino acids (GIBCO, 1% v/v), vitamins for minimum essential med-

ium (GIBCO, 0.4% v/v), sodium pyruvate (100 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), HEPES (10 mM) and human serum albumin (HSA; 1% w/v, Central Laboratory, Swiss Red Cross Blood Transfusion Service, Berne, Switzerland). After 6 to 24 h of culture in CO_2 atmosphere (5%) at 37 °C, supernatants were collected, pooled within groups and snap frozen in liquid nitrogen for TNF determination. The cells remaining in the plates received 100 µl of saline, and were used for PCA measurement, in microtiter plates immediately thereafter. In some experiments, monocytes were highly purified by centrifugation-elutriation, using a published procedure[19].

Monocytes contained in MNC were allowed to mature to macrophages under nonadherent conditions[17,20]. In brief, monocytes were purified by selective adherence to polystyrene, followed by dislodgement 18 h later. The cell suspension was cultured in teflon bags (DuPont de Nemours, type FEP 100 A) for 6 to 7 d of culture in CO_2 atmosphere (5%) at 37 °C without medium change. During this time, mature macrophages of a purity of >95% and a viability of >90% were recovered. They were washed twice with phosphate-buffered saline (PBS), resuspended in PCA medium and subcultured in microtiter plates (3×10^4 macrophages per well) for 6 to 24 h in the presenece or absence of LPS (*E. coli* O111:B4 and *E. coli* O55:B5, Sigma, St. Louis, MO).

The contents of TNF in mononuclear phagocyte supernatants (2-fold, 4-fold or 8-fold dilutions) was determined by a cytotoxicity assay, using murine L929 fibroblastoid cells as targets as described[21]. The activity was expressed as U/ml, using recombinant TNF-α (National Institute for Biological Standards and Control, Potters Bar, England) as a standard. PCA was determined by the recently described turbidimetric recalcification time assay[22] and expressed in thromboplastin units. One unit was arbitrarily defined as the amount of PCA corresponding to that of a 10^6-fold dilution of thromboplastin (Thromborel S, Behringwerke, Marburg, Germany). The turbidmetric version of the LAL assay was performed essentially as described[23]. The lysate (Haemachem Inc., St. Louis, MO) had a nominal sensitivity of 0.06 endotoxin units (EU) and could detect 0.016 endotoxin units per ml (1.6 pg/ml *E. coli* O55:B5), in our hands. Lipopolysaccharide-binding protein (LBP) was assayed by determining by flow-cytometry the association of FITC-labeled LPS with the monocyte surface[24].

Results

Monocyte responsiveness to LPS. Fresh MNC expressed little, if any, detectable PCA, and did not constitutively produce TNF. Upon exposure to LPS in microtiter plates, both PCA expression and TNF production were induced, and this response was dose and time-dependent (fig. 1). At least 4 h were required for induction of

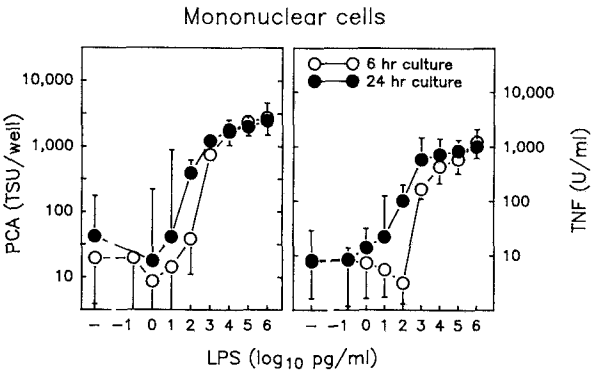

Figure 1. PCA expression and TNF production by MNC as a function of LPS concentration. Fresh MNC (3×10^4 monocytes/well) were cultured in microtiter plates for 6 to 24 h in the presence of the indicated concentration of LPS (*E. coli* O111:B4). After 6 to 24 h, supernatatants were collected for TNF determination, and adherent cells were used for PCA determination. Values refer to means ± SD of 5 independent determinations.

TNF and PCA detected by bioassays. The minimal stimulatory dose was between 10^2 and 10^3 pg/ml LPS (*E. coli* O111:B4) and between 10 and 10^2 pg/ml LPS for 6 and 24 h of stimulation, respectively (fig. 1). There was a considerable degree of inter-donor variation for both TNF and PCA production, but induction by 1 ng/ml or 100 pg/ml for 6 to 24 h, respectively, was significant ($p < 0.05$) in all instances, and reached a plateau at 10 to 100 ng/ml. For comparison, LPS *E. coli* O111:B4 yielded an accelerated coagulation in the LAL test at concentrations >/ = 10 pg/ml (not shown). Both PCA and TNF production by MNC were entirely due to monocytes, as demonstrated by the use of centrifugation-elutriation-purified cells (not shown). PCA was mediated to a large extent by cell surface-associated tissue factor, as reported elsewhere[9].

Macrophage responsiveness to LPS. The maturation of monocytes to macrophages in teflon containers was accompanied by significant changes in the in vitro PCA response to LPS. Constitutive (noninduced) PCA showed a sharp rise during the first 24 h of culture, and then increased more slowly during the subsequent 6 d (fig. 2). LPS-stimulated cells showed a significantly enhanced PCA when compared with nonstimulated cells cultured for the same duration. The stimulation index, i.e. the ratio between PCA of LPS-stimulated cells and control cells, was highest on stimulation of fresh cells and then slowly declined to values below 10 on stimulation at day 7 in the case of cells exposed to LPS for 6 h. In the case of cells exposed to LPS for 24 h, the stimulation index fell even more quickly to reach levels below 1 after day 4. The rapid loss of PCA by suspension-cultured, LPS-stimulated macrophages has previously been found to be associated with a state of unresponsiveness, in which cells are unable to express PCA on stimulation by the same or a different stimulus[25]. Thus, monocytes and macrophages differed significantly with respect to

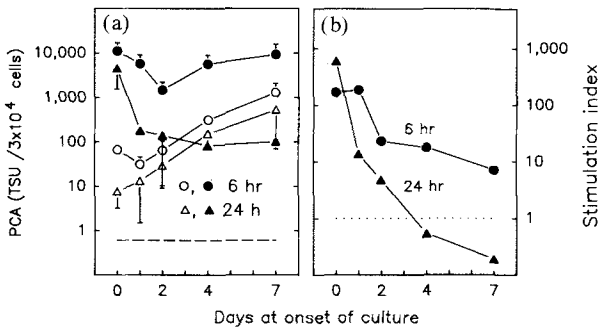

Figure 2. *A* Constitutive PCA (open symbols) and LPS-induced PCA (closed symbols) of fresh MNC and of monocytes cultured for various periods for time in teflon containers. The indicated times (days) denote the day at which cells were exposed to LPS for another 6 to 24 h prior to PCA determination. Generally, cells were cultured in polystyrene flasks for the first 18 h, a procedure used to separate monocytes from lymphocytes. Day 0 cells were cultured in microtiter plates only. Values represent means ± SD of 3 independent experiments performed with 3×10^4 monocytes/macrophages cells per well. Dashed line denotes PCA of freshly isolated, noncultured MNC (3×10^4 monocytes/well).
B Stimulation indices (PCA of LPS stimulated cells/PCA of nonstimulated cells cultured for the same interval) of the experiments of *A*. Dotted line (1.0) denotes lack of stimulation).

constitutive PCA expression (low in monocytes, high in macrophages), with regard to the LPS-induced increase in PCA (high in monocytes, low in macrophages), and with regard to subsequent decline in PCA (slow in monocytes, rapid in macrophages).

Another difference between monocytes and macrophages was revealed by dose-response experiments using macrophages exposed to LPS in an adherence type subculture (microtiter plates). Macrophages responded to at least 1000-fold lower LPS concentrations both by enhanced PCA expression and by augmented TNF production (fig. 3). The lowest stimulatory concentrations were 2 orders of magnitude lower than the LPS detection threshold of the LAL assay, as shown in figure 4 with LPS *E. coli* O55:B5.

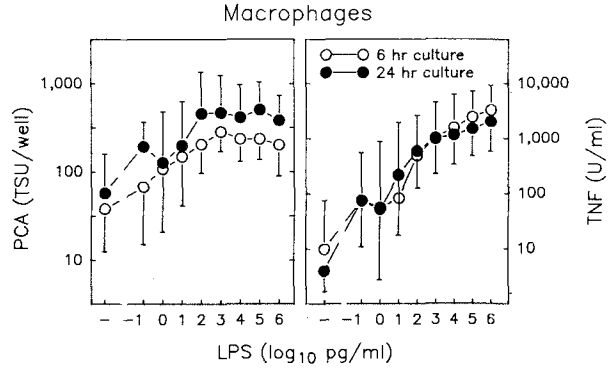

Figure 3. PCA expression and TNF production by 7-day-old macrophages as a function of LPS concentration. Macrophages (3×10^4 cells/well) were cultured in microtiter plates for 6 to 24 h in the presence of the indicated concentration of LPS (*E. coli* O111:B4). After 6 to 24 h, supernatatants were collected for TNF determination, and adherent cells were used for PCA determination. Values refer to means of 5 independent determinations.

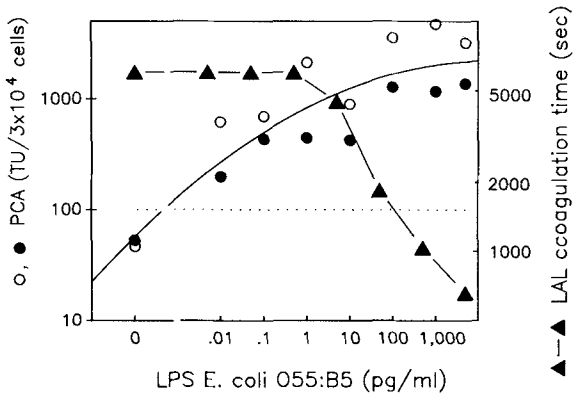

Figure 4. LPS-induced bioactivity as determined by the turbidimetric LAL assay (triangles) and by PCA induction in teflon-bag-cultured macrophages (circles). The same dilution row of LPS *E. coli* O55:B5 was tested in both assays. Each symbol represents the mean of triplicate determinations. Open and closed circles refer to 2 cell batches from 2 different donors. The dotted line (100 TU/10^4 cells) marks the threshold level of significance of this particular PCA assay.

The influence of various culture conditions on macrophage PCA and TNF production. Given the functional versatility of macrophages, the effect of changes in the culture conditions on LPS-induced PCA and TNF were explored. Monocytes purified by selective adherence were found to develop into macrophages expressing less PCA than monocytes purified by elutriation-centrifugation (not shown). Consequently, stimulation indices for LPS-stimulated cultures were lower in elutriated, differentiated macrophages than in adherence-purified, differentiated cells. This is in harmony with two recent studies using either elutriated[26] or adherence purified[25] monocytes. Cells subjected to elutriation, then to adherence-purification followed the pattern of non-elutriated, adherence purified cells (not shown). Other functional parameters, including TNF production, were not found to differ between the two populations of macrophages (not shown).

Interferon-γ (IFN-γ) and LPS were shown to synergize with regard to many macrophage functions, including TNF production and PCA expression[22,27–29]. Macrophages primed with 500 U/ml IFN-γ for 48 h in teflon bags, then subcultured in the presence of LPS, showed an increase in both PCA and TNF production when compared with unprimed counterparts; however, there was no further increase in sensitivity to low LPS concentrations by IFN-γ priming (not shown).

Macrophages exposed to LPS in suspension, in teflon bag culture, were compared with macrophages exposed to LPS in microtiter plate subcultures, containing either serum-free medium with HSA as a protein source, or 15% homologous serum (fig. 5). The same dose-response curves were obtained regardless of whether LBP was detectable (serum-containing medium) or not

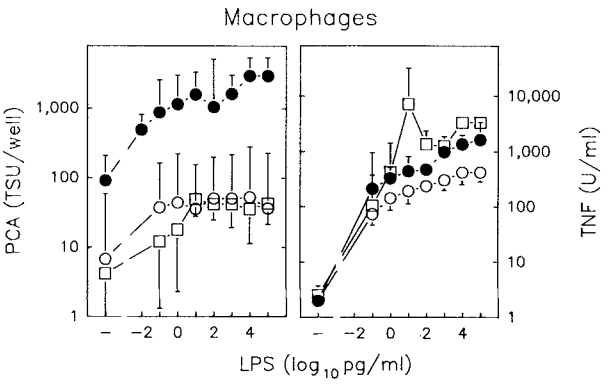

Figure 5. PCA expression and TNF production by macrophages (d7 to 8) exposed to varying concentrations of LPS (*E. coli* O55:B5) either in teflon bag suspension cultures (closed circles), or in microtiter plate subcultures (open symbols). Circles: medium containing 15% human AB serum; open squares: medium containing 1% HSA. Values refer to means ± SD of 5.

(HSA-supplemented medium). This suggests that the exquisite LPS sensitivity of macrophages did not involve lipopolysaccharide-binding protein (LBP). A moderate increase of PCA upon LPS stimulation was observed in the adherence subculture only; a larger increase was seen when cells were exposed to LPS directly in the teflon bag cultures (figs 4 and 5), suggesting that LPS in part superseded effects induced by adherence to polystyrene.

Discussion

The most significant findings of this study may be summarized as follows.

1) Macrophages exposed to LPS in HSA-containing medium were 2 orders of magnitude more sensitive than monocytes, and surpassed the detection sensitivity of the LAL assay >100-fold, regardless of whether PCA or TNF was considered. This behavior was observed under a variety of culture conditions and using different monocyte isolation methods.

2) Sensitivity of mature macrophages to low concentrations of LPS did not depend on the presence of serum factors in the fluid phase.

3) Due to the higher constitutive PCA level of macrophages, the stimulation index for LPS-induced PCA was much lower in macrophages than in monocytes, and was much lower than that for TNF induced in macrophages. Constitutive PCA expression by macrophages appeared to depend on factors other than LSP, e.g. on adherence to polystyrene, which is in line with earlier observations[30].

Both LPS recognition and LPS-induced signal transduction are only partially understood, and much of this knowledge is based on investigations of murine macrophages or monocytoid cell lines[1, 15, 16, 31–36]. An important pathway of monocyte/macrophage triggering by LPS is the LPB-CD14 pathway[13]. LPS complexed with LBP, but not free LPS, binds with high affinity to cellular CD14, a surface determinant expressed by both monocytes and macrophages. CD14 triggering is involved in the promotion of TNF production[24, 37]. In our study, macrophage sensitivity was not influenced by the presence of LBP in the medium, since the serum, but not the HSA used, contained LBP activity. Both monocytes and macrophages used expressed high levels of CD14, as determined by flow cytometry (not shown). In contrast, monocytes were reported to display significantly enhanced sensitivity to low LPS concentrations when LBP is present[24]. This suggests that macrophages have a mechanism for detecting low concentrations of LPS which is independent from the LPB-CD14 axis, and this mechanism promotes both tissue factor expression and TNF production. It is not known, however, whether the increase in sensitivity to low LPS concentrations during differentiation of monocytes to macrophages is due to altered recognitions, or to modified signal transduction. Similar LPS sensitivity studies have not been reported with cell lines, which would allow a closer analysis of the recognition and signal transduction mechanisms involved. The present evidence for a high affinity interaction of LPS with human macrophages in the absence of LBP indicates there are as yet unknown cellular binding sites for LPS expressed in mature macrophages.

The present study has two distinct types of methodological implications. The first concerns functional studies involving mononuclear phagocytes. An important consideration is whether these had been inadvertently stimulated by endotoxin either during cell isolation or cell culture, or by agents to which cells are exposed. Fetal bovine sera and demineralized water are the most frequent sources of contaminating pyrogens. The limits conventionally given for absence of pyrogen contamination (<1 EU/ml) are 3 to 4 orders of magnitude too high for excluding an endotoxin effect in human mononuclear phagocyte studies. Likewise, the inability of polymyxin B to reduce a response does not prove the absence of pyrogens, since the two functions tested here, TNF production and PCA expression, are both incompletely blocked by polymyxin B (T. W. Jungi, R. Miserez and I. Schwager, unpubl. observ.), and crude endotoxins might even be less susceptible to polymyxin B than the purified LPS species used here[38].

The second methodological implication is the use of mononuclear phagocyte type cells as indicators for endotoxin/pyrogen contamination. Current procedures either involve animal experimentation (rabbit pyrogen test) or make use of the LAL test. Given the phylogenetic distance to vertebrates, the LAL test may poorly reflect a pyrogen reaction in humans or other vertebrates, and substances found to be pyrogenic cannot always be detected by such procedures[39]. The use of monocytes[40, 41] or related cell lines[39, 42] may offer a possi-

bility to monitor for pyrogen contamination. Here, we clearly show that monocyte-derived macrophages surpass any other known pyrogen detection assay in sensitivity, including the one using monocytoid cell lines[39,42]. A combination of two simple assays, such as the presently used PCA screening test[22] and the widely used TNF determination, may be indicative for the presence of LPS, since both functions are induced by minute amounts of LPS and other pyrogenic agents, but are otherwise differently regulated[25]. Ongoing studies are aimed at selecting monocytoid human cell sublines which share with macrophages the exquisite sensitivity towards LPS.

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