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CD14 and tolerance to lipopolysaccharide: biochemical and functional analysis

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SUMMARY

To evaluate the role of the high-affinity monocyte receptor for lipopolysaccharide (LPS), CD14, in the process of tolerance to LPS, the human monocytic cell line Mono-Mac-6 was cultured in the absence or presence of different amounts of LPS. The kinetics of CD14 modulation in these cells showed an initial 4-day period characterized by increased cell-surface expression, rate of biosynthesis (peaking at 48 hr) and release of its soluble forms (sCD14) which correlated with the amount of LPS in the culture. At this time, tolerance to LPS was already established, as measured by tumour necrosis factor-α (TNF-α) induction, it was LPS dose dependent and persisted up to 15 days. LPS also reduced the cell proliferation rate in a dose-dependent manner. After 8 days and up to 15 days, the CD14 biosynthesis, cell-surface expression and release of sCD14 inversely correlated with the level of LPS in the culture. The 48-hr LPS-pretreated cells showed a slightly decreased CD14 affinity for LPS, a relative high number of CD14 molecules per cells, and desensitization also to a phorbol 12-myristate 13-acetate (PMA) challenge. An anti-CD14 monoclonal antibody (mAb) protected the cells from tolerization when added at the beginning of culture, as revealed by challenge with LPS and PMA. The data indicate that in this model tolerization to LPS (1) precedes CD14 down-modulation, (2) operates by alteration of the receptor affinity for LPS and by a mechanism which affects a protein kinase C (PKC)-dependent signalling pathway, and (3) that CD14 plays a critical role in the establishment of tolerance to LPS. In addition, analysis of the data suggests the existence of a PKC-independent signalling pathway for LPS tolerization and a CD14-independent mechanism for establishing tolerance.

INTRODUCTION

Monocytes/macrophages respond to a variety of stimuli including tumours and infectious agents by releasing a series of immunoregulatory and inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor-α (TNF-α).[1] Excessive secretion of these mediators of host response can cause deleterious effects including shock and death.[1,2] The excessive release of TNF-α by monocytes plays a major role in the pathophysiological effects caused by infection with Gram-negative bacteria or injection with lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria.[3–6]

Abbreviations: LPS, lipopolysaccharide; L, M, H medium, low-, medium- and high-LPS content culture medium, respectively; MFI, median fluorescence intensity; mCD14, membrane-bound CD14; mAb, monoclonal antibody(ies); PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; sCD14, soluble CD14; TNF-α, tumour necrosis factor-α.

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The regulatory mechanisms of the TNF-α synthesis and secretion are complex and not well defined.[7,8] In rabbits, for example, sequential intravenous injections of LPS administered at intervals of 5 hr, result in TNF-α release into the blood only after the initial LPS injection but not following a second LPS dose.[9] This phenomenon, defined as tolerance or desensitization to LPS,[10] has been the focus of several investigations.[9,11–16] Limited information, however, is available regarding the underlying molecular mechanism(s). It is known that LPS tolerance does not apparently involve a negative feedback by TNF-α or TNF-α-inducible cell products as it cannot be induced by infusion of recombinant TNF-α.[9] Moreover, prostaglandins, known to inhibit LPS-induced TNF-α production by macrophages, do not seem to be alone in playing a critical role in this phenomenon.[13–16] Furthermore, it was shown[12,14] that tolerance to LPS is characterized by a decrease in the LPS-induced TNF mRNA without modifications in its half-life, and appears to be LPS-specific as LPS-tolerant macrophages showed a normal TNF-α response to *Staphylococcus aureus*.[14]

The cells of the monocyte/macrophage lineage are the principal target for LPS and the main source of LPS-induced

TNF-α *in vivo*.[3,5] LPS binding to cell-surface receptor(s) constitutes the primary step in the signalling events that lead to the induction of TNF-α. The myeloid differentiation antigen CD14[17] was identified as the receptor for complexes between LPS and the plasma protein termed LPS-binding protein.[18,19] Moreover, it was shown that anti-CD14 mAb can block the TNF-α production in whole blood exposed to LPS concentrations in the range necessary to induce symptoms of sepsis.[18] These findings suggest that CD14 is a critical receptor involved in the monocyte responses to LPS. However, a study on the involvement of CD14 in the process of tolerance to LPS is still required. This issue was addressed in the study reported here by employing a human monocytic cell line.

MATERIALS AND METHODS

Antibodies

The CD14-specific mouse monoclonal antibodies (mAb) used were MEM-18 (IgG1κ),[20] purified from ascites fluid (provided by Dr V. Bazil, Czechoslovak Academy of Science, Praha, Czechoslovakia) by affinity chromatography on a protein G–Sepharose (Pharmacia/LKB AG, Duebendorf, Switzerland) column, and B-A8 (IgG1, Innotherapie Laboratories). Our unpublished results showed the specificity of B-A8 for CD14, as judged by immunofluorescence followed by flow cytometric analysis of peripheral blood mononuclear cells and immunoprecipitations followed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). This mAb, however, does not block the LPS-induced TNF-α release by Mono-Mac-6 cells (see Results and Fig. 3, 1 day). Rabbit anti-mouse immunoglobulin was from Nordic Immunology (Lausanne, Switzerland). Additional mAb used in immunofluorescence and flow cytometry included a phycoerythrin (PE)-labelled anti-CD14 mAb (Leu-M3, IgG2b),[17] a fluorescein isothiocyanate (FITC)-labelled anti-CD45 mAb (HLe-1, IgG1),[21] and labelled, isotype-matched controls (all from Becton Dickinson AG, Basel, Switzerland).

Cell culture

In this study the monocytic cell line Mono-Mac-6[22] (kindly provided by Dr L. Ziegler-Heitbrock, University of Münich, Germany) was used as an experimental model. Mono-Mac-6 typifies mature monocytes on the basis of morphological, functional and phenotypic criteria.[12,15,22] Cells were maintained in RPMI-1640 medium (Seromed, Biochrom AG, Berlin, Germany) supplemented with heat-inactivated 10% fetal calf serum (FCS) (Amimed AG, Muttenz, Switzerland), gentamicin (50 μg/ml; Gibco AG, Basel, Switzerland), non-essential amino acids (1% v/v; Gibco), insulin (9 μg/ml; Sigma Chemie Gmbh, Diesenhofen, Germany), oxalacetate (1 mM; Sigma), pyruvate (1 mM; Seromed) and L-acetyl-L-alanyl-L-glutamine (1% v/v; Seromed), thereafter referred to as complete medium, as described previously.[22] The culture medium was ultrafiltered through PM-10 filters (Amicon, Lausanne, Switzerland) prior to the addition of FCS. The LPS concentration in the complete medium was $\leqslant 35$ pg/ml, as determined by the Limulus amoebocyte lysate assay ('E-Toxate'; Sigma). The cells were routinely seeded at 1×10^5 cells/ml concentration in 24-well tissue culture plates (Nunc, Life Technologies AG, Basel, Switzerland). For kinetic studies of CD14 modulation and LPS tolerization (Figs 1–3), cells were seeded at a lower concentration, i.e. $0{\cdot}5 \times 10^5$ cells/ml and maintained in the indicated culture media (see Results) without feeding. When indicated, cell cultures were supplemented with *Escherichia coli* LPS, Serotype 055:B5 (Sigma) and/or anti-CD14 mAb. The cell viability prior to the experiments was always >95% as determined by trypan blue exclusion.

TNF-α induction assays and blocking experiments

Mono-Mac-6 monocytes were washed in cold LPS-free phosphate-buffered saline (PBS), resuspended at 1×10^6 cells/ml concentration in complete medium supplemented with various amounts of LPS and seeded (300 μl) in 5-ml polystyrene Falcon tubes (Becton Dickinson). Cell supernatants were collected after 2 hr incubation (optimal incubation time, determined in preliminary experiments) at 37°, centrifuged and tested for TNF-α by enzyme-linked immunosorbent assay (ELISA) ('Quantikine', R&D systems, Bühlmann Lab, Basel, Switzerland).

For some experiments, the TNF-α induction assays were performed in the absence or presence of the anti-CD14 mAb (16 μg/ml) MEM-18, known to block the LPS-induced TNF-α and IL-6 release by monocytes, or B-A8, isotype-matched control, non-blocking antibody (unpublished observations).

TNF-α induction assays with phorbol 12-myristate 13-acetate (PMA) were performed as described for LPS. Cells were resuspended in complete medium supplemented with increasing concentrations of PMA (Sigma; stock solution of 1 mM PMA in dimethylsulphoxide).

Immunofluorescence and flow cytometry

Cells were washed, adjusted to 4×10^6 cells/ml concentration in PBS, and then incubated with normal rabbit serum for 10 min at room temperature. Subsequently, cells (2×10^5) were incubated in the presence of PE-labelled anti-CD14, FITC-labelled anti-CD45 or control mAb for 15 min at room temperature. After washing, cells were resuspended in PBS/1% paraformaldehyde and examined for median fluorescence intensity (MFI) by using a FACScan flow cytometer (Becton Dickinson) and acquiring at least 10,000 cells/sample.

Cell-binding saturation assays

Mono-Mac-6 cells maintained in the different culture medium (see Results) were washed three times in cold PBS and 2×10^5 cells were incubated for 30 min at room temperature in complete medium supplemented with increasing amounts of FITC–LPS (*E. coli*, serotype 055:B5; Sigma), and 0·2% v/v sodium azide, maintaining the final volume at 200 μl. The reaction was stopped by addition of 4 ml of cold medium/0·2% sodium azide, and the cells were washed for 1 min. Subsequently, the cells were fixed with PBS/1% paraformaldehyde and the bound material was then analysed for MFI by flow cytometry. Specific binding was obtained by subtracting from each binding value the non-specific binding, which was defined as the linear arm of the total-binding plot.

The cell-binding saturation data were analysed by using an equation related to that of Scatchard.[23] The derived equation was: $B/T = KB_{max} - KB$, where B = bound ligand (MFI arbitrary units); T = total ligand concentration (μg/ml); B_{max} = maximal bound ligand at saturation, and K = affinity constant (ml/μg). It

was not possible to determine the maximal fluorescence of the ligand in our experimental system, and the unknown degree of aggregation of LPS in aqueous solution precluded its MW definition. Thus, the total number of receptors per cell and the K values were not determined. However, the slope of the B/T ratios versus B plot and the extrapolation to abscissa permit a relative estimation of K and total number of receptors, respectively. Data analysis was performed by using a BMDP program (BMDP statistical software, University of California Press, CA).

Cell labellings and preparation of supernatants

Mono-Mac-6 cells were surface-labelled with Na ^{125}I (Amersham International, Amersham, U.K.) by the lactoperoxidase-catalysed iodination technique[24] at room temperature for 45 min. Cell viability after labelling was greater than 95%. For analysis of the sCD14 release, surface-labelled cells were washed in cold medium, resuspended in prewarmed medium (supplemented with different amounts of LPS) at a concentration of 2×10^5 cells/ml and incubated for 5 hr at 37°. Subsequently, equal numbers of viable cells (3×10^6) were collected. Cells and

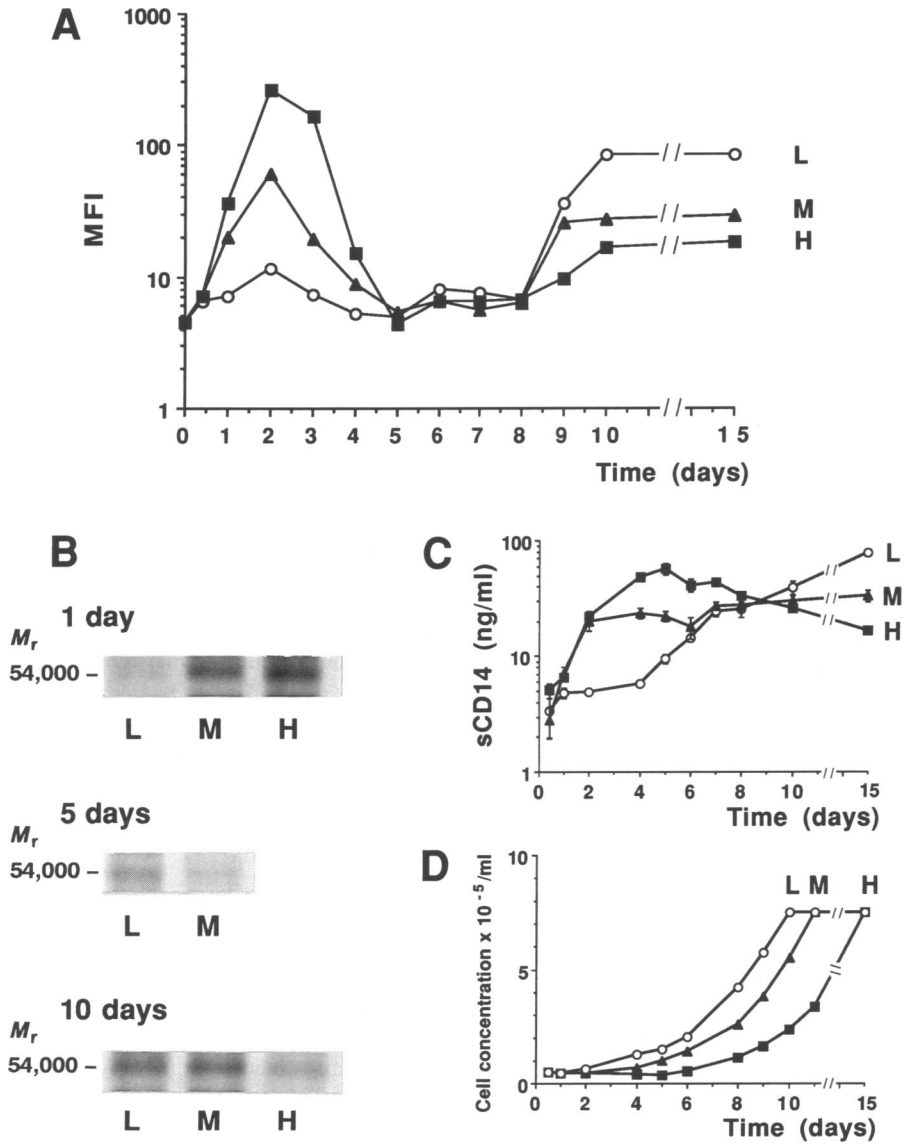

Figure 1. (A) Time-course of the LPS-induced modulation of the CD14 expression in Mono-Mac-6 monocytes cultured in low-LPS-content medium (L) alone or supplemented with additional 20 pg/ml (M) or 200 pg/ml (H) LPS. CD14 cell-surface expression at the culture times indicated was assessed by examining cells for MFI by flow cytometry. Data, expressed in arbitrary units, represent the mean of one representative experiment out of three performed in duplicate. (B) Membrane-bound CD14 biosynthesis by Mono-Mac-6 cells cultured in L, M or H medium. At the time-points indicated, cell aliquots were collected, biosynthetically labelled, and the cell lysates tested for CD14 reactivity by immunoprecipitation followed by SDS–PAGE analysis. M_r estimates were based on M_r markers. (C) Time-course of the release of sCD14 by the Mono-Mac-6 cells cultured as described in (A). Concentrations of sCD14, secreted by 1×10^5 cells, were determined by ELISA. Data represent the mean of two experiments. (D) Cell proliferation rate of the L-, M- and H-medium cultured cells during the 15-day study. The number of viable cells in each cell culture was determined at the indicated time-points. Values represent the mean of four independent determinations (SD $\leq$ 10%).

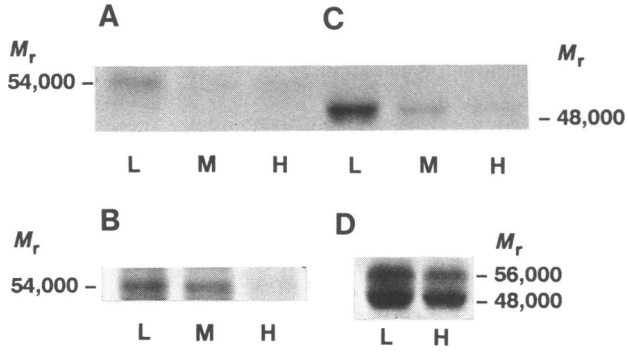

Figure 2. Expression of mCD14 (A and B) and sCD14 (C and D) in Mono-Mac-6 monocytes cultured for 15 days in low-LPS-content culture medium alone (L) or supplemented with additional 20 pg/ml (M) or 200 pg/ml (H) LPS. The cells were surface labelled and recultured for 5 hr (A and C) or biosynthetically labelled for 17 hr (B and D). The cell lysates and supernatants were tested for mCD14 and sCD14 reactivity by immunoprecipitation followed by SDS–PAGE analysis. M_r estimates were based on M_r markers.

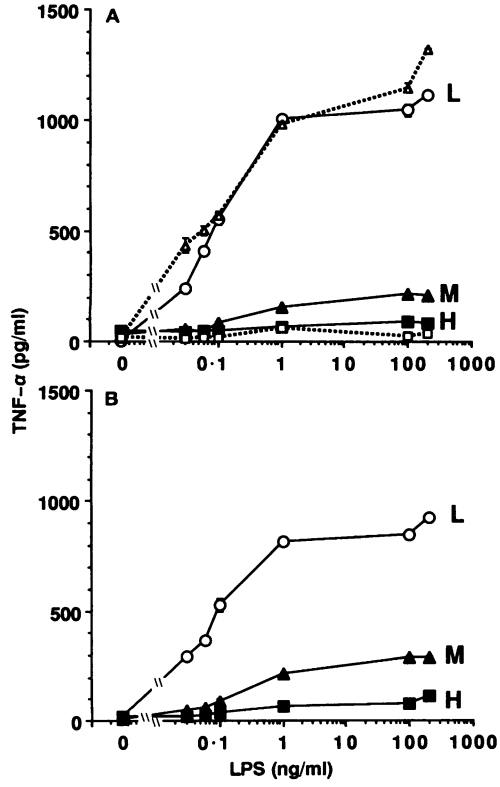

Figure 3. Dose–response curves showing the levels of TNF-α secreted by Mono-Mac-6 cell aliquots (3×10^5 cells) taken after 1 (A) and 2 (B) days from cultures with low content of LPS (L) or supplemented with additional 20 pg/ml (M) or 200 pg/ml (H) LPS and challenged for 2 hr with the indicated amounts of LPS. After incubation, supernatants were tested for TNF-α by ELISA. Dotted lines correspond to dose–response curves performed in L-medium cultured cells in the absence or presence of a CD14-specific mAb known to block the LPS-induced TNF-α release by monocytes [MEM-18 (□)], or a non-blocking anti-CD14 mAb [B-A8 (△)] used as isotype-matched control. Data represent the average of three independent experiments with SD (vertical bars).

supernatants were separated by centrifugation at 4°. Cells were washed in ice-cold PBS containing 0·02% (w/v) sodium azide and the cell lysates prepared immediately. The supernatants were filtered through 0·22 μm filters (Amicon) and ultracentrifuged (100,000 *g*, 30 min at 4°). Nonidet P-40 detergent (NP-40; BDH, Poole, U.K.) and phenylmethylsulphonylfluoride (PMSF; Sigma) were then added at final concentrations of 0·5% (w/v) and 2 mM, respectively. The supernatants were then desalted in Centricon-10 microconcentrators (Amicon) with PBS/0·02% sodium azide and lysis buffer [10 mM Tris–HCl, pH 7·4, 150 mM NaCl, 0·5% (w/v) NP-40, 0·02% (w/v) sodium azide, 2 mM PMSF], and concentrated 10 times for the immunoprecipitation experiments.

Mono-Mac-6 cells were biosynthetically labelled with a 17-hr pulse of [^{35}S]methionine (Amersham) as previously described.[25] The labelling medium, i.e. minimal essential medium (MEM) lacking L-methionine (Gibco), was supplemented with the amounts of LPS indicated in Results. For analysis of the sCD14 release, the culture supernatants of the biosynthetically labelled cells were recovered and prepared as already described.

Immunoprecipitations and SDS–PAGE analysis

The labelled cells were lysed (1×10^7 cells/ml of lysis buffer), centrifuged at 4°, and the cell lysates subjected to immunoprecipitations as previously described.[26] Cell lysates and concentrated supernatants were precleared with a solution of protein A-bearing *Staphylococcus aureus* Cowan I (SAC-I, ICN Immunobiologicals, Lisle, U.K.) and SAC-I conjugated to rabbit anti-mouse immunoglobulin (SAC-I-RAM). The precleared material was incubated with MEM-18 mAb (3 μg) for 30 min at 4°. The immunocomplexes were precipitated with SAC-I-RAM. The analysis of the immunoprecipitates was performed under reducing conditions in 10% SDS–PAGE according to the method of Laemmli.[27] The gels were fixed, dried, fluorographed in Amplify (Amersham) and autoradiographed at −80° using Hyperfilm MP films (Amersham) with intensifying screens.

Estimations of sCD14 concentrations

The sCD14 concentration in cell culture supernatants was estimated by a sCD14-specific ELISA (IBL, Hamburg, Germany).

RESULTS

Kinetics of LPS-induced CD14 modulation

It has previously been shown that LPS may induce up- or down-modulation of CD14 cell-surface expression in monocytes and monocyte-derived macrophages.[28 30] Thus, we first tested the pattern and kinetics of the LPS-induced modulation of CD14 in the Mono-Mac-6 cell line. The cells were cultured in culture medium with a low LPS content (≤35 pg/ml LPS) alone or supplemented with additional 20 pg/ml or 200 pg/ml LPS which we refer to as low (L)-, medium (M)-, or high (H)-LPS-content medium, respectively. At various time-points cells and culture supernatants were collected. Cells were tested for mCD14 expression and biosynthesis (Fig. 1A and B, respectively), and the culture supernatants were tested for sCD14 release (Fig. 1C).

Flow cytometric analysis (Fig. 1A) showed an LPS dose-dependent up-regulation of mCD14 between the first and fourth day of culture, with a peak at 48 hr. The CD14 up-regulation at

this stage was IL-6 mediated, as an anti-IL-6 mAb prevented it (data not shown), confirming previous observations.[31,32]

Comparative SDS–PAGE analysis of the 54,000 MW mCD14 polypeptides immunoprecipitated with a CD14-specific mAb from [^{35}S]methionine-labelled cells, showed that the initial LPS dose-dependent mCD14 over-expression was accompanied by a higher rate of mCD14 biosynthesis (Fig. 1B).

The release of sCD14[33,34] from the Mono-Mac-6 cells showed an overall correlation with the mCD14 biosynthesis and cell-surface expression (Fig. 1C). The time–course of the sCD14 release showed, however, that the variations in mCD14 cell-surface expression preceded those in sCD14 concentrations.

The LPS dose-dependent increased expression of CD14 during the initial period of the study was observed in spite of the higher rate of cell proliferation showed by the L-medium cultured cells (Fig. 1D).

To test whether LPS affected further the expression of its receptor, we continued monitoring the kinetics of CD14 expression up to 15 days of cell culture in the different media. The initial period was followed by 4 days during which there were no significant differences in mCD14 expression among the L-, M- and H-medium cultured cells. After 8 days of culture, and up to 15 days, the L-medium cultured cells showed the highest mCD14 expression, whereas the cells cultured in M and H medium showed intermediate and the lowest mCD14 expression, respectively (Fig. 1A). It was possible that the decreased mCD14 expression in the LPS-treated cells resulted from a reduced rate of its synthesis or defective translocation to the plasma membrane, resulting in the intracellular accumulation of the receptor. After 5 days of culture, however, the rate of mCD14 biosynthesis also showed an inverse correlation with the LPS level in the culture medium and this pattern was maintained after 10 days (Fig. 1B). An increased release of sCD14 may also account for the decreased mCD14 expression. However, the variations in mCD14 biosynthesis and cell-surface expression were followed by similar changes in the sCD14 release (Fig. 1C).

The expression of the leucocyte common antigen CD45, simultaneously monitored, did not show any LPS-mediated variation in the three cell preparations studied (not shown). It should also be noted that the fluorescence histograms showed that all cells responded in a similar manner to LPS with respect to the up- and down-modulation of CD14 expression. Indeed, the pattern of CD14 staining covered a wide range of fluorescence intensities (> 1 log) and it was shifted uniformly to higher or lower values upon LPS induced up- or down-modulation respectively (data not shown).

To study in more detail the relative down-regulation of CD14 by prolonged exposure to LPS, Mono-Mac-6 cells maintained for 15 days in the three different culture media were surface labelled and subsequently recultured for 5 hr in the same medium. Cells and supernatants were then tested for CD14 reactivity by immunoprecipitation followed by SDS–PAGE analysis (Fig. 2). The intensity of the 54,000 MW mCD14 polypeptide band inversely correlated with the LPS content in the corresponding culture medium (Fig. 2A). The difference in mCD14 expression was greater between cells cultured in L medium and those cultured in M or H medium than between the latter two.

The intensity of the biosynthetically labelled 54,000 MW mCD14 polypeptide band also showed an inverse correlation with the LPS content of the corresponding culture medium (Fig. 2B). Analysis of the culture supernatants of the surface-labelled cells showed strong, weak and barely detectable 48,000 MW sCD14 polypeptide bands, corresponding to the mCD14-derived sCD14 released by L-, M- and H-medium cultured cells, respectively (Fig. 2C). In addition to the 48,000 MW sCD14 form, Mono-Mac-6 cells, as well as normal human monocytes, release a 56,000 MW sCD14 form detectable in culture supernatants of biosynthetically but not surface-labelled cells (M. O. Labeta, J.-J. Durieux, N. Fernandez, R. Herrmann and P. Ferrara, submitted). Thus, to examine whether the secretion of both sCD14 forms was affected by the culture conditions, the culture supernatants of biosynthetically labelled cells which had been cultured in L and H medium were tested for sCD14 expression. Figure 2D shows that the amount of both the 56,000 and 48,000 MW sCD14 forms released from the H-medium cultured cells was reduced, as judged by comparison of their SDS–PAGE patterns to those corresponding to the L-medium cultured Mono-Mac-6 monocytes. A quantitative estimation of the effect of the culture conditions on sCD14 release by 15-day cultured Mono-Mac-6 cells showed that cells cultured in L medium release 2·4 and 4·9 times more sCD14 than those cultured in M and H medium, respectively. LPS does not seem to interfere with the detection of sCD14 by ELISA because: (1) we did not find significant differences in concentration when highly purified 48,000 MW sCD14, diluted in any of the three

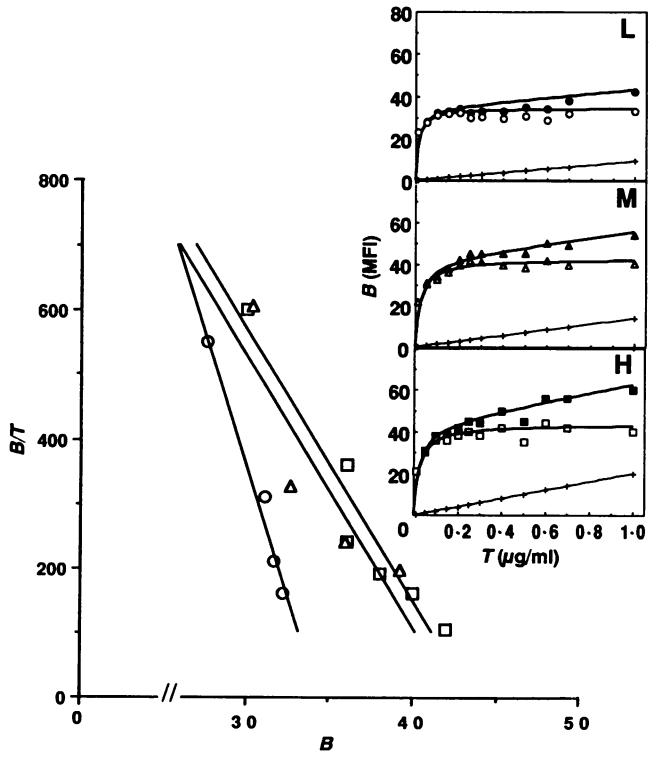

Figure 4. Cell-binding saturation assays in 48-hr L (circles)-, M (triangles)- and H (squares)-medium cultured cells (2×10^5 cells) performed as described in Materials and Methods. The binding data were plotted as binding of FITC-LPS (*B*) as a function of FITC-LPS concentration (*T*) (inset), or as *B*/*T* ratios versus *B* by an equation related to that of Scatchard. Open symbols in both plots indicate specific binding. Closed symbols and crosses in the *B* versus *T* plots indicate total and non-specific binding, respectively. Data represent the average of four independent experiments with SD $\leqslant$ 10%.

culture media described here, was measured by ELISA either before or after 24 hr incubation at 37°; (2) when similarly tested, culture supernatants of 4-day L-medium cultured Mono-Mac-6 cells, supplemented or not with additional 20 pg/ml or 200 pg/ml LPS, showed similar values of sCD14 concentration (not shown).

Kinetics of cell desensitization to LPS

Next, we evaluated the functional effect of the initial LPS-induced mCD14 up-regulation on the tolerization phenomenon. Cell aliquots were taken after 1 and 2 days of culture in the three different media. After washing, the cells were stimulated with varying amounts of LPS for 2 hr before the determination of TNF-α release (Fig. 3). The dose–response curves showed that despite the marked up-regulation of mCD14 in the H- and M-medium cultured cells during this period, the L-medium cultured cells were the highest responders to LPS doses up to 200 ng/ml. The Mono-Mac-6 cells maintained in M medium showed hyporesponsiveness, whereas the H-medium cultured cells were not sensitive to the LPS doses tested. This dose–response pattern was maintained during the 15-day period of the study (not shown).

Additional TNF-α induction assays in L-medium cultured cells were performed in the absence or presence of a CD14-specific mAb known to block (MEM-18) or not (B-A8) the LPS-induced TNF-α release by monocytes (Fig. 3, 1 day). The MEM-18 mAb but not B-A8 (isotype-matched), blocked the release of TNF-α by cells stimulated with up to 200 ng/ml LPS concentrations, indicating that LPS stimulation of Mono-Mac-6 in the different culture media operates via CD14 only.

CD14 binding affinity for LPS in the L-, M-, and H-medium cultured cells

The LPS-dependent desensitization of the cells to further stimulation with LPS, even during the initial period of mCD14 up-regulation already described, could be the result of a decreased affinity of CD14 for LPS. We, therefore, performed a comparative analysis of the receptor–LPS interaction affinity in 48-hr L-, M- and H-medium cultured cells (Fig. 4). The data of cell-binding saturation with FITC–LPS (Fig. 4 inset) when plotted as B/T ratios versus B showed a linear fit of the experimental points. Comparison of the slope of this plot for each cell preparation revealed a twofold bigger slope for the L-medium cultured cells as compared to that of the M- and H-medium cultured cells. No significant differences in the slope were observed between the latter two cell preparations. As the binding of FITC–LPS to the cells could be inhibited by MEM-18 mAb (not shown), the results suggested that the affinity constant for the CD14–LPS interaction in the L-medium cultured cells is slightly higher than that of the other cell

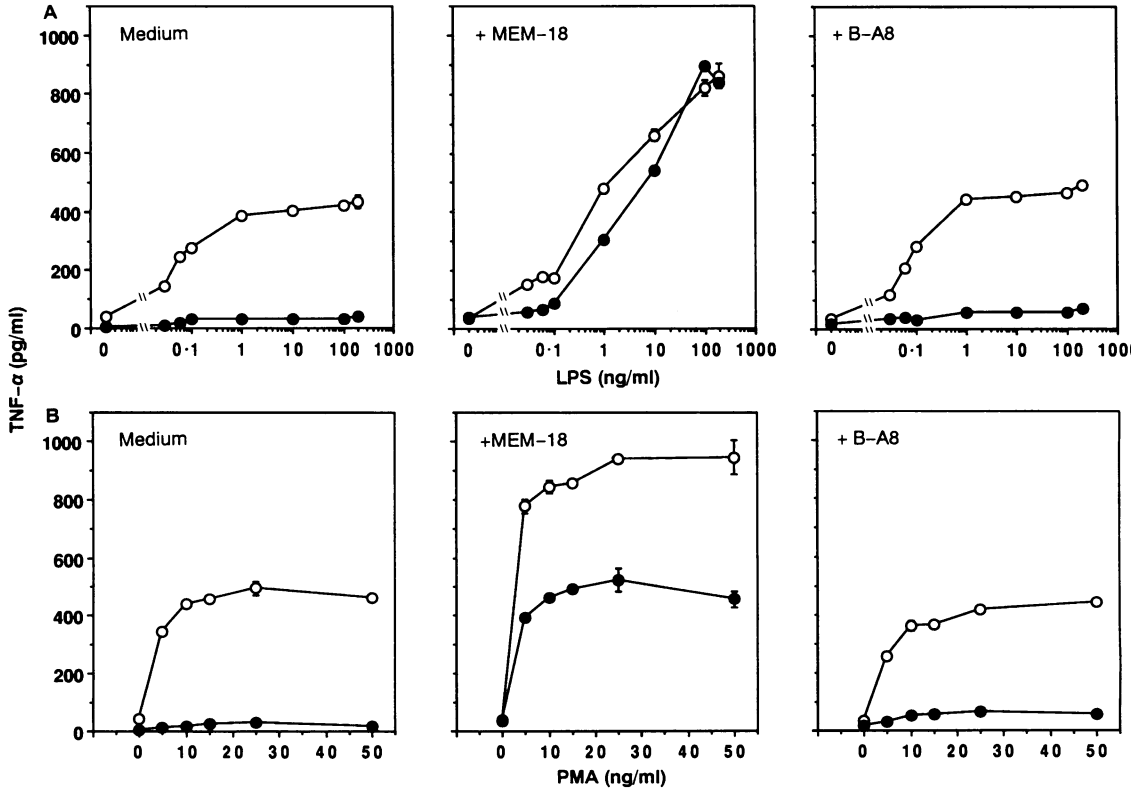

Figure 5. Dose–response curves showing the levels of TNF-α released by Mono-Mac-6 cells (3×10^5 cells) when challenged with the indicated amounts of LPS (A) or PMA (B). The cells were cultured for 48 hr in low-LPS-content culture medium (○) alone or supplemented with additional 200 pg/ml LPS (●), in the absence or presence of an anti-CD14 mAb (16 μg/ml) known to block the LPS-induced TNF-α release by monocytes (MEM-18) or a non-blocking anti-CD14 mAb (B-A8) used as isotype-matched control. Subsequently, the cells were washed and challenged for 2 hr with the indicated reagent. Data represent the average of three experiments with SD (vertical bars).

preparations tested. The limitations of the experimental procedure did not permit calculation of the absolute values of K or the number of LPS receptors in each cell preparation. However, a comparative estimation of the latter parameter from the cell-binding saturation plots and by extrapolating to the abscissa in the B/T versus B plots, indicated that the H- and M-medium cultured cells expressed significantly more LPS receptors per cell than the L-medium cultured cells. This result is in agreement with the FACS and biochemical analyses shown in Fig. 1.

Response of LPS-pretreated cells to PMA

Next, we asked if desensitization to LPS affected the capacity of the cells to secrete TNF-α by a receptor-independent mechanism. As LPS seems to act, at least in part, through activation of protein kinase C (PKC),[6] 48-hr L- and H-medium cultured cells were challenged with increasing amounts of the PKC activator PMA. Figure 5B (left panel) shows that the H-medium cultured cells completely lost the capacity to secrete TNF-α upon stimulation with PMA doses up to 50 ng/ml.

Role of CD14 in the establishment of tolerance to LPS

To evaluate the role of CD14 in the establishment of desensitization to LPS, cells were cultured in L or H medium for 48 hr in the absence or presence of the CD14-specific mAb MEM-18 (blocking mAb) or B-A8 (non-blocking mAb). Subsequently, the cells were washed and challenged with either LPS or PMA (Fig. 5A or B, respectively). The MEM-18 but not B-A8 mAb was able to protect the cells from tolerization to further stimulation with LPS (Fig. 5A). In the case of the L-medium cultured cells, this effect was observed at high LPS stimulatory doses, i.e. > 1 ng/ml LPS. In the H-medium cultured cells, the MEM-18 protective effect was observed at high as well as low LPS stimulatory doses. At low doses, however, the protective effect on this cell preparation was partial when compared to that of L-medium cultured cells in the presence or absence of mAb. Analysis of the cell response to challenge with PMA (Fig. 5B), revealed a drastic protective effect of MEM-18 on the L- and H-medium cultured cells in all the ranges of PMA concentrations tested.

DISCUSSION

In this study we used a human monocyte-like cell line to evaluate the involvement of CD14 in the process of tolerance to LPS. It has been suggested that a reduction in the number of LPS-specific monocyte receptors may be responsible for this phenomenon.[11,14,16,35] However, the analysis of the kinetics of CD14 modulation (Fig. 1) and tolerance to LPS (Fig. 3) in the Mono-Mac-6 cell line showed that desensitization to LPS precedes an LPS-induced late CD14 down-modulation. Indeed, tolerance to LPS was already established by the time the cell-surface expression of CD14 in the LPS-treated cells was maximal, i.e. in the initial 4-day period. Only prolonged exposure of cells to LPS led to a dose-dependent relative down-modulation of both mCD14 and sCD14 when compared with control cells (L medium). This may indicate that, at this time, the turnover rate of CD14 in response to low levels of LPS (< 35 pg/ml) is higher than that in response to higher levels (up to 235 pg/ml). This notion is supported by the high rate of CD14 biosynthesis shown by the L-medium cultured cells as compared to that of M- and H-medium cultured cells (Fig. 1B, 10 days; Fig. 2B and D). The LPS effect seems to be additional to an overall increase in CD14 expression observed in the three cell preparations over the time, probably related to physiological changes as the age of the culture increased.

Studies in other receptor–ligand systems showed that desensitization may also operate by a decreased receptor-binding affinity for the ligand.[36] This factor seems to operate in the CD14–LPS system, as the cell-binding saturation assays shown in Fig. 4 revealed that the 48-hr cultured LPS-treated cells, although expressing significantly more LPS receptors per cell, bind LPS with a twofold lower affinity compared to control cells. This slight alteration of the receptor affinity for the ligand, however, is not alone responsible for the tolerization phenomenon. Indeed, the response of LPS-tolerant cells to the PKC activator PMA was found to be completely suppressed (Fig. 5B, left panel), indicating that desensitization to LPS involves alteration of a PKC-dependent signalling pathway. This conclusion is consistent with the report of Haas *et al.*[15] who concluded that desensitization to LPS operates at a transcriptional level by a mechanism that involves PKC. Nevertheless, CD14 seems to be critically involved in the establishment of tolerance, as judged by the protective effect of the MEM-18 mAb on the cell response to LPS and PMA, when added at the beginning of culture. The protective effect of the mAb on the L-medium cultured cells was detectable only at relatively high LPS challenging doses, whereas in the H-medium cultured cells a partial protective effect was observed at low doses of LPS, and a response similar to that of control cells was observed at higher doses. The response of the cells at low LPS challenging doses might be affected by the blocking effect of the mAb still bound to the cells during the TNF induction assays.

Careful comparison of the cell response to LPS and PMA (Fig. 5A and B) shows that the effect of MEM-18 mAb on the H-medium cultured cells was partial when tested by PMA challenge; the response did not parallel that of the control cells, as it did when cells were challenged with LPS (compare cell response in Fig. 5A with B, middle panels). This observation is consistent with the existence of a CD14-independent mechanism for establishing tolerance. As anti-CD14 mAb can block completely the response to LPS, as shown in Fig. 3, the postulated CD14-independent mechanism would involve LPS uptake by the cells and transmission of only a negative signal affecting PKC. Evidence supporting this mechanism has recently been reported.[37] The partial effect of the mAb on the H-medium cultured cells observed by PMA stimulation, could also be explained as a result of displacement by LPS during the 48 hr incubation. This seems unlikely in view of the excess of mAb as compared to LPS in the H medium (16 μg/ml mAb versus ⩽235 pg/ml LPS). Moreover, MEM-18-treated L- and H-medium cultured cells showed similar responses at high-LPS stimulatory doses (Fig. 5A, middle panel).

The different responses of the H-medium cells to treatment with MEM-18 as evaluated by challenge with PMA and LPS may indicate that partial restoration of the PKC-mediated signalling pathway is sufficient to allow an efficient response to LPS via CD14. However, the cells cultured in L medium without mAb showed a lower response to challenge with high doses of LPS than MEM-18-treated H-medium cultured cells, in spite of the fact that both cell preparations showed a similar response to

PMA [compare the response of MEM-18-treated H-medium cells in Fig. 5A (middle panel) with that of L-medium cells in Fig. 5A (left panel) and that of the same cell preparations in Fig. 5B (middle and left panels, respectively)]. Taken together, these observations are also consistent with the existence of a PKC-independent pathway of LPS stimulation via CD14.

In summary, several conclusions emerge from our study. (1) LPS tolerance is already established by the time LPS induces maximal up-modulation of its high-affinity receptor, CD14. (2) Only prolonged stimulation of cells with LPS leads to a relative down-modulation of CD14, as compared to control cells. Thus, this effect may contribute to but is not the primary event in monocyte desensitization to LPS. (3) Tolerization operates by alteration of the receptor affinity for LPS and by a mechanism which affects a PKC-dependent signalling pathway. (4) CD14 plays a critical role in the establishment of tolerance to LPS. In addition, the data suggest (5) the existence of a PKC-independent pathway of LPS stimulation via CD14 and a CD14-independent mechanism for establishing tolerance.

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