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Coordinate up- and down-modulation of inducible nitric oxide synthase, nitric oxide production, and tumoricidal activity in rat bone-marrow-derived mononuclear phagocytes by lipopolysaccharide and gram-negative bacteria

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Simultaneous incubation of primary rat bone-marrow-derived mononuclear phagocytes (BMMø) and tumor cells with gram-negative agents triggers within 24 h interferon γ (IFNγ)- and tumor necrosis factor (TNFα)-independent tumoricidal activity. On the other hand, BMMø that had been incubated for 24 h with gram-negative agents prior to re-exposure to the same agent had largely lost their ability to generate tumoricidal activity, although their ability to bind lipopolysaccharide (LPS) was not diminished. Parallel measurements of the kinetics of inducible nitric oxide synthase (iNOS), nitrite secretion, and tumoricidal activity triggered in primary BMMø by LPS revealed that these parameters take a coordinate course, reaching a peak within 24 h and then rapidly decaying.Down-regulation of expression of NOS protein and iNOS activity could be attributed neither to down-regulation of LPS receptors nor to L-arginine depletion.

Mononuclear phagocytes (monocytes, macrophages, histiocytes, Kupffer cells, etc.) play an essential role in defense against microbial agents and neoplasia (1). Bacteria and several of their products are potent of inducing in a pure lymphocyte-free population of rat BMMø a secretory (tumor necrosis factor, TNFα; nitrite, NO^-_2) and cellular response (mitochondrial respiration; IFNγ- and TNFα-independent tumoricidal activity; 2-4). BMMø that were first incubated for 24 h with gram-positive bacteria and then interacted with tumor targets in the continuing presence of the same bacteria expressed marked tumoricidal activity (2,5). Under the same conditions, gram-negative bacteria, lipopolysaccharide (LPS), and lipid A were poor

Abbreviations: BMMø, bone marrow-derived mononuclear phagocytes; iNOS, inducible nitric oxide synthase, IFNγ, interferon γ; LPS, lipopolysaccharide; NFκB, nuclear factor κB; NO, nitric oxide; NO^-_2, nitrite; TNFα, tumor necrosis factor.

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inducers of tumoricidal activity (2,3). In the present study it is shown that gram-negative agents trigger in primary BMMø a biphasic response: initial activation is rapidly passing over to an inactive and unresponsive condition; iNOS, NOS protein, nitrite secretion, and tumoricidal activity took a corresponding course.

MATERIAL AND METHODS

Materials

Most chemicals and other reagents were from Sigma Chemical Co., St. Louis, MO. LPS from *Escherichia. coli* 055:B5 and its FITC conjugate were from List Biological Laboratories, Campbell, CA; tetrahydrobiopterin was from Dr. B. Schircks, Jona, Switzerland; rat rIFNγ was from Dr. P.H. van der Meide. Bacteria were selected, grown, harvested, and heat-inactivated as described (2); wet weight was taken as a measure of their amount.

Methods

Bone marrow-derived mononuclear phagocytes (BMMø). Bone marrow cells from femurs of inbred barrier-raised male Zur:SIV (Sprague-Dawley) rats were cultured in Iscove's modified Dulbecco medium supplemented with 5% fetal calf serum and conditioned with supernatant (final concentration 10%) from strain L clone 929 cells as described (5). On day 6 after initiation, BMMø remaining adherent after repeated washing were incubated for the time interval indicated in IMDM medium containing the usual amount ($5x10^{-4}$ M) or a surplus of L-arginine ($9x10^{-4}$ M; 6) and supplemented with one of the agents under test before their functional activities were determined.

Flow cytometry. BMMø were incubated for 24 h in medium alone or in medium supplemented with LPS, *Moraxella. catarrhalis*, or *E. coli*, washed twice, incubated for 60 min at 4°C with LPS-FITC (1 μg/ml), washed, and fixed with paraformaldehyde (1%, 20 min) before analysis by flow cytometry (7).

NO^-_2 determination. Cell-free sample aliquots were mixed with Griess reagent, incubated for 20 min at room temperature, and nitrite concentration determined by measuring A_{550} (2).

NO synthase activity (L-citrulline assay). Aliquots of macrophage lysates (100'000 g x 1 h supernatants; 30 μg protein) were incubated in the absence or presence of N^G-nitro-L-arginine (L-NNA, 1 mM) for 10 min at 37°C with 1 μM [^{3}H]-L-arginine (1 μCi), 1 mM NADPH, 15 μM tetrahydrobiopterin, 1 μM flavine adenine dinucleotide, and 1 μM calmodulin in 50 mM Hepes buffer, pH 7.4, containing 1 mM dithiothreitol, 1 mM EDTA, and 1.25 mM $CaCl_2$ (final volume 150 μl). Incubation was terminated by the addition of 1 ml cold 100 mM Hepes buffer, pH 5.5, containing 10 mM EGTA and 500 mg Dowex AG 50W-X8 (counter-ion Na^+) cation exchange resin (Serva). After incubation at 0 to 4°C for 5 min, and centrifugation at 10'000 x g, [^{3}H]-L-citrulline was quantified by liquid scintillation counting; iNOS activity was calculated as the L-NNA-sensitive formation of [^{3}H]-L-citrulline per minute and mg protein.

Detection of immunoreactive protein of NOS (Western blot). Aliquots (20 μg protein) of 100'000 g x 1 h supernatants and sediments of cell homogenates were electrophoretically separated on 8% SDS-polyacrylamide gels and blotted on nitrocellulose. NOS was specifically detected by a polyclonal rabbit antibody against iNOS expressed in RAW 264.7 cells (M. Richards, University of Michigan, Ann Arbor). Immunoreactive protein was made visible by the ECL method (Amersham).

Tumoricidal activity was used as the primary measure of macrophage functional activity (5). Two different experimental conditions were examined: 1) resting

BMMø were incubated for 24 h with activating agent and targets; 2) BMMø were first incubated for 24 h with activating agent, the medium then replaced by new medium supplemented with the same or another activating agent and target cells, and incubated for a further 24 h. TNFα-resistant P-815 mastocytoma cells that had been prelabeled with [^{14}C]-thymidine were utilized as target cells (effector/target cell ratio 2.5:1 and 5:1); after incubation, radioactivity in cell-free supernatants was measured and the percentage of specific thymidine release was calculated (5).

RESULTS

The macrophage response to bacterial agents differs depending on the experimental conditions. Simultaneous incubation with tumor cells and gram-negative agents triggered in rat BMMø marked tumoricidal activity (Fig. 1A). An inverse response was obtained when BMMø were first incubated for 24 h with gram-negative agents, the medium then replaced by fresh medium supplemented with the same stimulant and target cells (Fig. 1B). Such down-regulation of the macrophage reactivity after incubation with gram-negative agents was seen in the large majority of experiments (2,3), quite irrespective of whether the medium contained the usual concentration or a surplus of L-arginine (data not shown; 6). To identify the causes responsible for the diminished reactivity of macrophages after incubation with gram-negative agents, various approaches were made.

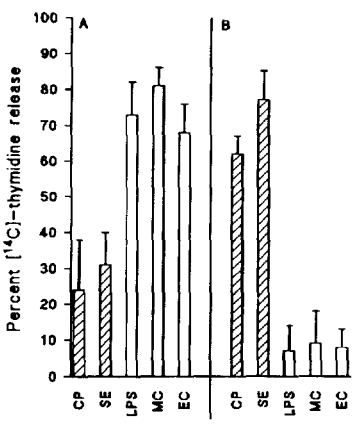

Fig. 1. The capability of macrophages to generate tumoricidal activity in response to gram-positive (▨) and gram-negative (□) bacterial agents differed depending on the experimental conditions. **A.** Simultaneous incubation for 24 h of BMMø, bacterial agents and prelabeled tumor cells. **B.** BMMø were incubated for 24 h with bacterial agents before the medium was replaced by fresh medium supplemented with the same bacterial agent and tumor cells; incubation was for a further 24 h. Initial effector/target cell ratio 2.5:1. Values are the means (± SD) from four to five experiments, each performed in triplicate. Concentrations of bacterial agents: *Corynebacterium parvum*, CP, 25 μg/ml; *Staphylococcus epidermidis*, SE, 25 μg/ml; LPS,5 ng/ml; *M. catarrhalis*, MC,2.5 μg/ml; *E. coli*, EC, 10 μg/ml.

Binding of LPS is not diminished by pretreatment of macrophages with gram-negative agents. The ability of macrophages to bind LPS was not diminished by treatment with gram-negative agents (Fig. 2).

Incubation of macrophages with gram-negative agents affects induction of iNOS, NO^-_2 secretion, and tumoricidal activity in a corresponding manner. In further experiments, the kinetics of the various macrophage activities was determined. After simultaneous incubation of BMMø with LPS and tumor targets, expression of NOS protein (Western blot) and iNOS activity (L-citrulline formation) in cytosolic extracts, nitrite secretion and tumoricidal activity took a coordinate course, reaching peak activity within 24 h but rapidly decaying within the following 12 h (Fig. 3). Labeling of immunoreactive NOS protein was approximately 5-fold more intense in the cytosol than in the particulate subcellular fraction, as assessed by densitometry. In both fractions, NOS followed a similar time course (Fig. 3). Pretreatment of BMMø with gram-negative agents for 24 or 36 h, but not for 6 h, also correspondingly reduced the capability of BMMø to generate the secretory and cell-mediated activities in the course of subsequent incubation with the same or another gram-negative agent, as exemplified for LPS and tumoricidal activity (Fig. 4). Similarly, preincubation for 24 h of BMMø with gram-negative agents also reduced the capability of BMMø to respond to renewed incubation with an increase in iNOS activity and nitrite secretion (data not shown).

DISCUSSION

Previous work has shown that rat BMMø that had first been incubated for 24 h with gram-positive bacteria expressed considerable cytolytic activity when directly afterwards incubated with tumor cells in the presence of the same bacteria (2,5).

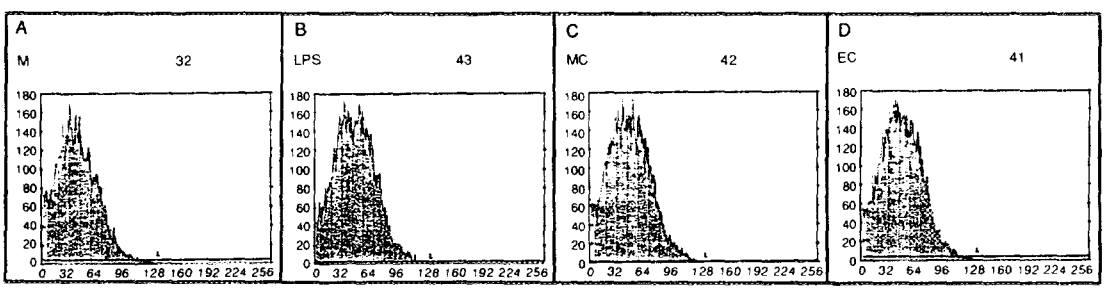

Fig. 2. Incubation for 24 h with LPS (B), *M. catarrhalis* (C), or *E. coli* (D) did not diminish the ability of BMMø to bind FITC-conjugated LPS. A = control, medium alone (M). Concentrations of agents were as in Fig 1. Values represent mean fluorescence intensity.

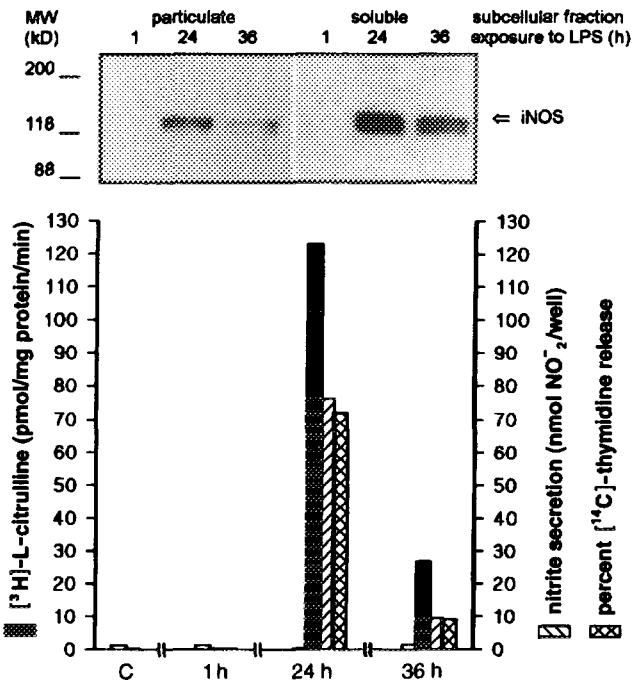

Fig. 3. Incubation with LPS triggers in BMMø a transient expression of iNOS and tumoricidal activity. BMMø were incubated without (control) or with LPS (5 ng/ml) for 1, 24, and 36 h (exchange of medium and LPS after 24 h). For the determination of NOS protein and iNOS activity, the cells were homogenized, and soluble (100'000 g x 1 h supernatant) and particulate (100'000 g x 1 h sediment) fractions were analyzed separately for immunoreactive NOS protein (Western blot, upper part). iNOS activity ([^{3}H]-L-citrulline formation) was determined in the soluble fraction.

Under the same conditions, LPS and most gram-negative bacteria triggered in BMMø at best limited tumoricidal activity (2,3). As shown in Fig. 1, simultaneous incubation of BMMø, bacterial agents, and tumor cells for 24 h resulted in an inverse response: gram-negative agents induced high tumoricidal activity while gram-positive bacteria were in the majority of experiments clearly less active. To identify the mechanisms underlying the diminished macrophage reactivity following preincubation with gram-negative agents, various attempts were made. LPS has been shown to reduce the responsiveness of macrophages which was sometimes associated with diminished binding of LPS (8,9). As binding of LPS was not diminished by pretreatment with gram-negative agents (Fig. 2), the macrophage hyporesponsiveness is unlikely to be due to down-regulation of receptor expression.

Evidence is still increasing that generation of nitric oxide from L-arginine and expression of microbicidal and tumoricidal activities by macrophages are closely

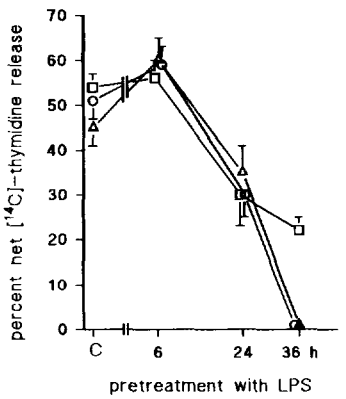

Fig. 4. The ability of primary rat BMMø to generate tumoricidal activity in response to gram-negative agents was progressively diminished as pretreatment with LPS was prolonged. BMMø were first incubated for 6, 24, or 36 h with LPS (5 ng/ml) before their 24 h interaction with prelabeled P-815 mastocytoma cells (initial effector/target cell ratio 2.5:1) in the presence of LPS (5 ng/ml; O), *M. catarrhalis* (2.5 μg/ml; Δ), or *E. coli* (10 μg/ml; □). Controls (C) were directly incubated for 24 h with target cells in the presence of these bacterial agents. Values are the means (± SD) from five experiments, each performed in triplicate.

related processes (10). Pre-exposure to LPS has been shown to inhibit induction of iNOS by IFNγ in the murine macrophage cell line, J774 (11), and to inactivate iNOS activity in primary mouse peritoneal macrophages (12). Moreover, iNOS from a rat alveolar macrophage cell line was inhibited by nitric oxide (13). On the other hand, incubation with LPS or gram-negative, but not gram-positive bacteria, triggered in primary rat BMMø in the absence of IFNγ within 24 h high iNOS, the secretion of large amounts of nitrite, and marked tumoricidal activity (14). With the present demonstration that the initial increase in these macrophage activities triggered by LPS is rapidly followed by their simultaneous decay, resulting in diminished ability to respond to LPS and to gram-negative bacteria, we have come to come full circle. The mechanism of action that accounts for the down-modulation of iNOS expression in BMMø by gram-negative agents is unknown. Tolerance to TNFα gene transcription induced by pretreatment of human monocytic MonoMac 6 cells with LPS is likely to be a consequence of LPS-triggered synthesis of a functionally inactive NFκB complex (15). Since NFκB controls transcriptional activation of the iNOS gene in response to LPS in many cells (16,17), it may well be that a similar mechanism acounts for the only transient expression of iNOS reported here.

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